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CONTENTS

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CONTINUOUS FLUIDIZED BED GASIFICATION OF CELLULOSIC WASTES FOR POWER GENERATION

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ABSTRACT

A continuous fluidized bed reactor system was developed for the gasification of cellulosic wastes for the production of gas-fuel product suitable for power generation. A practical operating procedure for steady state operating conditions for the conversion system was established. Based on the results obtained, the yield, gas composition and heating value of the gas products were found to be primarily influenced by such factors as feedrate, chemical composition of the raw materials, types and ratio of fluidizing medium and fluidizing gas used. The experimental results presented herein have established that low calorie gas products can be continuously produced from the gasification of cellulosic wastes using either steam, air or a combination thereof. Gas products with heating values ranging from 1,000 to 3,800 kcal/Nm^3 were obtained at optimal conditions.

Fluidized bed gasification system using air as fluidizing gas was found to be a practical process for the production of low calorie gas fuel which can be used for power generation as shown by the results obtained from the application testing of the gas products. Gas products with heating value as low as 1,100 kcal/Nm^3 proved suitable to fuel a gas engine for generation of electricity or for heat co-generation systems.

INTRODUCTION

Cellulose is the most abundant renewable resource on earth, either as plant material or as recyclable waste. Two types of cellulosic substances can be used as feedstock for thermochemical processing (pyrolysis and gasification), namely: 1) cellulosic by-products

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arising from the production of other materials and 2) cellulosic wastes generated from industrial and community activities (Ghose, 1978). These cellulosic materials, with chemical composition $(C_2H_{10}O_5)n$, can be decomposed at high temperatures into H_2, CO, CO_2 and hydrocarbon gases by adapting advance energy conversion system similar to the process utilized for fossil fuels like coal.

The Philippines is endowed with rich natural resources and has an abundant supply of cellulosic waste materials. The volume of these wastes emanating from urban, industrial or agricultural activities is increasing steadily in our country. These wastes have been disposed primarily by landfilling, burning or by discharging into the sea. These disposal methods have inherent air and water pollution problems but potentially valuable and recoverable energy resources remains unharnessed. With the shortage of fossil fuel supply and since proven reserves were declining, it is believed that cellulosic materials could play an increasingly important role as an alternative and renewable source of energy.

Gasification of solid fuels such as coal, biomass, etc. have been studied and applied more than a century ago (Kunii, 1969; Andum, 1977; Humphrey, 1978; Eather, 1980; Kawabata, 1979). Recent interest in producing fuels from renewable resources has focused on the use of lignocellulose as possible feedstock. Fuel gas production by thermochemical gasification of biomass and other solid fuels are expected to penetrate the energy market in the near future.

Literature reports (Beck, 1980; Van Swaaiji, 1981; Kohan, 1982; Mubaji, 1982) reveal that gasification is one of the four processing methods of energy recovery from agricultural and forestry residues and urban wastes presently being studied in response to the worldwide search for substitutes for petroleum based fuels. Clarke *et al* (1978) proposed gasification of crop residues which holds the greatest potential for alleviating natural gas curtailment to irrigators in the Great Plain States.

Thus, in response to the need to diversify energy sources in addition to alleviating waste disposal problems and thereby conserve natural resources, the National Institute of Science and Technology in a joint research venture with the Government Industrial Development Laboratory, Hokkaido, Japan, conducted a comprehensive five-year joint research study to develop an appropriate process for the conversion of urban and industrial organic wastes into usable forms of hydrocarbon products (such as fuel oil and gas) by applying pyrolysis and gasification technology adaptable to Philippine conditions.

Basic studies on the pyrolysis and subsequently gasification of copra, cassava bagasse, sawdust, coirdust, etc. by batch-fluidized bed method showed that the yield of liquid fuel, gas and char is greatly dependent on the bed temperature (NIST/GIDLH Final Report, 1985). Pyrolysis of solid wastes at lower temperatures (300-550°C) produced mainly liquid products whereas high temperatures (700-900°C) yielded mainly gas products. The liquid products obtained from pyrolysis however, constitute about 80% of water. Its removal would involve additional operating conversion process before the desired products could be recovered. As a result of the evaluation of the pyrolysis process, studies were then shifted to fluidized bed gasification at higher temperatures using

steam and air as fluidizing medium to convert the cellulosic wastes under study into gaseous fuel and char.

The laboratory phase gasification experiments carried out have established that such cellulosic wastes can be converted into fuel gas which possesses relatively high heating value ranging from 2,690 to 3,100 kcal/Nm^3. Results of tests done indicated that the gas product obtainable is combustible and exhibits red to bluish flame without smoke. It was found promising for use as household fuel and for various industrial applications like heat co-generating systems (Cruz, 1972; Miura, 1982).

This study then, was focused on the following objectives: 1) scale-up studies on the gasification of selected cellulosic waste materials for the production of gas product suitable for power generation and 2) application tests of the gas product obtained to generate electricity using a gas engine.

MATERIALS AND METHODS

1. Raw Materials

The selected cellulosic waste materials used for the gasification studies were industrial wastes which are generated in abundant quantities namely: sawdust, copra meal, coirdust and cassava bagasse.

1. Sawdust – The generation of this wood waste amounts to approximately two million tons annually from the production of logs, lumber and veneer in the Philippines. The sawdust used in the experiments consisted mostly of red lauan wood. The sawdust material was screened to remove big particle sizes. Sizes passing thru 16 mesh were used in the experiments.

2. Copra meal – The residue resulting from the extraction of oil from dried coconut meat (copra) was procured from the Philippine Refining Co., one of the biggest oil millers and edible oil producers in the Philippines. The meal was pre-treated by passing through a crushing machine to reduce the particle size to 0.5-1.0 mm.

3. Cassava bagasse is the residue left after processing cassava crop into starch. It was procured from La Peña Starch Manufacturing, one of the local flour millers located in San Carlos City. This plant generates about five (5) tons of waste bagasse per day.

4. Coirdust which constitutes 60-70% of the coconut husk is the waste produced after the extraction of fiber from the husk. It was procured from Coirflex Decorticating Company in San Pablo City, Laguna which accumulates about 8 M tons of coirdust per day. From day to day operations, it piles up into small mountains and its bulkiness and combustibility pose space shortage and fire hazard problems.

LPG with a heating value of 26,000 kcal/Nm^3, nitrogen gas with 99% purity and air were used in the preliminary tests using the gas engine.

2. Analyses

The physical properties of the raw materials and sand for the fluidized bed employed in the experiments were determined. Screen analysis and bulk density were also done. Heating value of raw materials was measured. The qualitative analysis of gas product was carried out using gas chromatography. The heating value of gas product was also calculated.

Analytical procedures adapted are in accordance with Japan Industrial Standard or with slight modifications thereof. Ignition tests on the gas products were conducted from time to time to check the spontaneity of gas flammability by lighting it with a match.

3. Equipment and Experimental Methods

3.1 Gasification Apparatus

The bench scale gasification apparatus used in this study is shown schematically in Fig. 1. The reactor (114 mm I.D. by 720 mm L) is made of stainless steel. It is equipped with a perforated plate having an opening ratio of 1.4% with hole diameter of 1.5 mm. The bed of the reactor is composed of sand with static bed height of 300 mm. The upper part of the perforated plate of the reactor (about one meter long) is heated by four semi-cylindrical heaters, two of which have the capacity to deliver 2 kwh and the other two with 1.5 kwh each. The heater has the capacity to increase the temperature at the rate of 12°C/min.

Steam as the fluidizing gas was generated from a coiled stainless steel pipe located at the upper part of the reactor. Heat evolved from the gasification reaction served as the heating medium to generate steam. A water roller pump with variable flowrate controller was used to force water through the coiled stainless steel pipe. The generated steam then passed thru the insulated pipe heated with electric heating wire.

Air as another fluidizing gas that was employed was furnished by a blower. It was charged at the wind box to serve as air preheating zone. The sample was first placed in a hopper which had a stirrer to avoid bridging of raw materials. The sample was charged continuously into the reactor by a screw feeder. The gas evolved from the reactor was allowed to pass through two cyclones where entrained fines were separated from the gas. The gas steam leaving the cyclone was cooled through a water spray at the top of the first cooling column. Mist, tar and water, which were removed by gravity were accumulated in the settling tank. A pump was used to circulate the water from the settling tank to the top of the spray tower. The scrubbed

gas then passed through a series of air-cooled pipes provided with fins where remaining tar and aqueous liquor were further removed. The volume of the resultant gas product was measured and then burned in the burning chamber before being allowed to emit into the atmosphere to avoid pollution.

The temperature was monitored by means of thermocouples placed at different parts of the apparatus. Temperatures were recorded graphically by a recorder and the pressure drop was monitored by means of water and mercury manometers.

The reactor containing a measured amount at a certain height of sand used as bed was initially pre-heated to the desired temperature using an external heater at a definite air flowrate ratio to sand. During this period, the steam temperature was brought to its operating temperature. The air flowrate was replaced with steam for steam gasification. Charging of waste materials into the reactor was done continuously at selected feedrates. Once the desired gasification temperature was attained and a steady flow was established, the time was set and gasification starts.

Gas sampling for analysis was done every 10 and 30 minutes under steady state operating conditions. The gas flowrate of the gas product was measured by means of a gas meter every 15 minutes and at the end of the operating time. The same procedure was adapted in the gasification experiments using air alone and a combination of air and steam as the fluidizing medium.

3.2 Gas Engine

The gas engine used in the application studies done on gas fuel products obtained consisted of two major parts: 1) a single cylinder, 4-cycle, 2.2 ps., and 143 cc gasoline engine known as a Robin Engine, manufactured by Fuji Heavy Company, and 2) a generator manufactured by Nishi Nippon Company. The parts were assembled by Kimmon Company based on the results of experiments they conducted. Some modifications were introduced to fully convert the engine from gasoline to gas fuel, the ignition timing was also altered. Final adjustments needed for the gas engine to run on city gas with a heating value of 5,000 kcal/Nm^3 were made before it was delivered for use in the gas application tests in the laboratory.

The converted engine, known as gas engine Model HG-1500, has the following specifications: voltage – 100 volts; power output – 1.1 kw and overall efficiency – 28%. An electric heater and an electric cutter with 300 w: 600 w up were used as the load during the test runs.

Further modifications and adjustment were made as needed on the gas engine during the test runs.

The gas engine was attached to the modified gasification apparatus. A scrubber was installed to remove CO_2 and facilitate the elimination of moisture from the gas products. The whole set-up is shown in Figure 2.

RESULTS AND DISCUSSION

1. Gasification Studies

The properties of the raw materials used as analyzed are shown in Table 1. Results of proximate and ultimate analyses showed that the waste materials contained significant organic components (hydrocarbon elements) similar to such fuels as coal, except that said wastes have smaller amounts of O_2 and moisture contents. On the other hand, cellulosic wastes have smaller amount of sulfur content and have lower ash content than coal. The heating value of the raw materials ranged from 4,000 to 4,800 cal/g. The bulk density of sawdust-B is high due to its high moisture content, 38%.

Results of the screen analyses of the samples, as shown in Figure 3 indicated that copra meal and cassava bagasse have almost the same average particle size of 1.2 mm; 0.7 mm for coir dust, sawdust-A and B; and 0.4 mm for sawdust-C.

For the initial experiments, Toyoura sand was used as fluidizing medium but the particle size was rather small that the sand passed thru the over-flow pipe. This contributed to the difficulty initially encountered in obtaining stable gasification temperature.

In view of this, bigger particle sizes of silica sand were used instead for the gasification experiments using steam. At temperatures above 500°C, it was found that the silica sand tended to melt and formed agglomerates which reacted with the residual charcoal. Under this condition, it was not possible to maintain steady state gasification operation. To eliminate such situation, olivine sand, which is composed mostly of MgO, was utilized instead. This sand was employed in subsequent gasification experiments using air and steam-air mixture respectively, as the fluidizing gases.

Screen analysis of the different types of sands used are graphically presented in Figure 4. The properties as analyzed are summarized in Table 2.

1.1 Gasification with steam

Initially, the necessary operating conditions that influence steady state fluidized bed gasification operation were investigated and established. Gasification studies on the different selected raw materials were subsequently conducted individually by varying bed temperature from 760 to 910°C and at established constant feedrate and steam flowrate using silica sand as fluidized bed.

The relationship between bed temperature and volume of gas product is shown in Figure 5. The trend clearly shows that the volume of gas product was greatly influenced by bed temperature as exhibited by all four raw mate-

rials. Gas product yields per kg of dry raw materials obtained at 800°C were as follows: sawdust, 1.2 Nm^3; coirdust, 0.9 Nm^3; cassava bagasse, 1.6 Nm^3; and copra meal, 1.1 Nm^3.

The gas composition of the product obtained varied with bed temperature as shown in Figures 6, 7, 8 and 9. Results indicated that both sawdust-A and cassava bagasse exhibited the same trend for individual gas component concentration. H_2 and CO_2 predominated at lower temperatures while CO predominated at higher temperatures. No significant increase in the concentration of CH_4 was observed. However C_2H_4 increased slightly with bed temperature, N_2 which was generated from the raw materials occurred in small concentration in the gas product. For coirdust and copra meal, a reverse trend in the gas concentration was observed. H_2 and CO_2 concentration in both gas products predominated at higher temperatures. Hydro-carbon gases such as CH_4, C_2H_4 decreased slightly with increasing bed temperature. The yields of combustible gas component obtained from the gasification of the different raw materials at 800°C, ranged from 80 to 83% for sawdust, cassava bagasse and copra meal, and was 74% for coirdust.

Each raw material produced gases which showed different trends in heating value with bed temperature as shown in Figure 10. Both sawdust-A and cassava bagasse produced gas products that exhibited higher heating values of 3,480 and 3,260 Kcal/Nm^3 at 900° and 800°C, respectively. Gas products obtained from coirdust and copra meal both exhibited the same decreasing trends in heating value beyond 743° and 850°C, respectively.

The heating value however of the produced gases can be increased by as much as 15% to 20% if the CO_2 content could be scrubbed from the fuel gas products. Using steam as the fluidizing medium, gas with 4,000 Kcal/Nm^3 which is adaptable for gas engine to generate energy and power could be obtained.

1.2 Gasification using a combination of steam and air

Studies on the gasification of sawdust-B and coirdust waste materials using air-steam mixture were done to determine the ideal operating conditions and variables to produce gas products suitable for generating power. The same gasification set-up was used as that for the steam gasification study, except that the location of the coiled stainless steel pipe for generating steam was readjusted by raising it to about 25 cm from its original position. Also an additional trap for tar collection was introduced in the set-up. Experimental runs were conducted at varying feed rates, bed temperatures, air to steam ratio and other variables that influence the gas product composition. Experiments using set variables were evaluated interrelatedly as presented in Figure 11.

Superficial velocity ratio of air and steam was varied from 1:1 to 1:2.5 to determine their effect on the quality of the gas product. At a ratio of 1:1 air to steam ratio, it was noted that the gas contains 51% N_2 from both coirdust and sawdust at a feedrate of 5 kg/h. Nitrogen is an inert gas with zero heating value and its presence in high concentration can greatly reduce the heating value of the gas product. Heating values of 1,350 Kcal/Nm^3 were obtained from coirdust and sawdust-A, respectively.

At a ratio of 1:2.5 air to steam, N_2 content was reduced to 41% at a feedrate of 5.9 kg/h using sawdust-B raw material. Gas product with heating value of 2,000 Kcal/Nm^3 was obtained. To reduce further the amount of nitrogen content in the gas product, the same ratio of air to steam (1:2.5) with added O_2 (1.2 cm/sec) was used. Results showed that nitrogen concentration in the gas product was reduced from 51 to 15% at the same feedrate of 5.9 kg/h. Presence of more O_2 in the gasification process resulted in higher CO_2 formation. The produced gas has almost the same heating value as that of the previous run wherein no O_2 gas was added. Based on the data and results obtained, air to steam ratio of 1:2.5 was found ideal to yield gas product with heating value of 2,000 Kcal/Nm^3.

For suitable operating conditions, it was found that the injection of a small amount of O_2 can minimize the supply of external heat. Besides, the temperature could be controlled and maintained easily and steadily.

The product yield was found to be influenced by the feedrate as shown in Figure 11. It was noted that the volumetric rate of gas produced (QT) increased with increasing feedrate.

The gas composition was evidently affected by the feedrate of 1 to 4 kg/h; a significant increase in gas concentration of H_2, CO, CH_4 showed only a slight change or almost attained equilibrium. For CO_2 concentration, a slight decreasing trend was noted. Absence of O_2 at higher feedrates showed that the O_2 in the fluidizing gas and from the sawdust material itself reacted with the char. For N_2, a reverse trend was noted with increasing feedrate. N_2 predominated at lower feedrates.

Heating value was also found to be influenced by the feedrate. Figure 13 indicates an increasing trend in heating value with feedrate. For sawdust it was found that a feedrate of 5.9 kg/h and gasification temperature of 800°C or above were ideal to generate gas product with heating values higher than 1,500 Kcal/Nm^3. Beyond these conditions, no significant increase in heating value was observed.

Gas product at a feedrate of 4.2 kg/h and above exhibited spontaneous flammability when ignited with a match. For ideal operating conditions, a lower feedrate of up to 5.9 kg/h had to be adapted to attain steady state operation. Beyond this point, the bed temperature could hardly be controlled steadily.

1.3 Gasification with air

Cassava bagasse, copra meal and sawdust were used in this study to determine the ideal operating conditions involved in the production of low calorie gas acceptable for heating purposes. Experimental runs were carried out using the same set-up. The external heater was used for the start up and when the bed temperature reached 300°C or more, raw materials were fed into the bed. Once the gasification temperature was attained, the external heater was put out. The heat evolved during the reaction between air and charcoal was sufficient to maintain the desired gasification temperature, thus a self-maintaining process was attained.

Feedrate was varied to determine its effect on bed temperature, quality, yield and heating value of the gas products at constant air velocity. Olivine sand was used as the fluidizing sand for all the experimental runs.

The relationship between feedrate and bed temperature is presented in Figure 14. It is clearly seen that bed temperature varies inversely with feedrate. At lower feedrate, bed temperature increased until a saturation point at 853°C was reached. At this point, reaction occurred resulting to almost complete combustion between the carbon in the sawdust and O_2 in the air. When the feedrate was increased beyond 0.7 kg/h, partial combustion occurred; thus the temperature tended to decrease. Flammability tests were performed on the gas products individually at increasing feedrates. Gas products obtained at lower feedrates were not flammable due to their high N_2 content. Flammable gas product was obtained at a minimum feedrate of 2.2 kg/h with spontaneous bluish flame.

It is evidently shown that the volume and heating value of the gas products increase with feedrate as exhibited by all three raw materials studied shown in Figures 15 and 16. For sawdust C, beyond 1.6 kg/h, no significant change in the concentrations of H_2, CO_2, C_2H_6 was noted. For CO and C_2H_8, a slight increasing trend was observed with increasing feedrates as shown in Figure 17. For both cassava bagasse and copra meal, an increasing trend for CO, H_2, CH_8 was noted with increasing feedrates. CO predominates the combustible gas products obtained. H_2 decreased with increasing feedrates. No significant change in CO_2 concentration was noted with feedrate as shown in Figures 18 and 19. The effect of feedrate on the heating value is shown in Figure 16. It was evident that heating value increased with feedrate

Experimental runs were conducted to determine the effect of O_2 at varying flowrates on the composition, heating value and yield of gas product using sawdust-C. As shown in Figure 20 no significant change in gas yield was noted with increase in O_2 flowrate. As shown in Figure 21, a slight change in trend for the individual gas components was observed. H_2, CH_4, CO showed almost the same concentration with O_2 flowrate. For N_2 a decreasing trend was however noted. CO_2 predominated at higher O_2 flowrates.

A higher heating value of 1,880 Kcal/Nm^3 was obtained at an O_2 flowrate of 4 L/min, which is higher than that obtained without the addition of O_2 as shown in Figure 20.

2. Application studies of the gas fuel product on the gas engine for power generation.

The operating conditions established in the bench-scale gasification studies using cellulosic waste materials were utilized in carrying out experimental runs to produce gas fuel to test its applicability for power generation. All runs were initiated by starting up using the established procedure for the air-blown gasification process at an air to feed ratio that was established for each waste material. These conditions were found to produce gas of low calorific value. Air and feedrates were adjusted to the desired operating conditions and allowed to approach steady-state.

The gas steam leaving the cyclone was cooled by allowing it to pass through a countercurrent water spray chamber. Fine charcoal, tar and water were removed and discharged at the drain. The gas was then passed through a CO_2 stripper using 15% NaOH solution as scrubbing liquid and then through a series of air cooled pipes with fins for further cooling, and finally through a filter column containing glass wool and silica gel to remove moisture and other impurities. The clean gas product was directly introduced into the gas engine. Gas samples were collected at this point for gas composition determination and heating value analysis.

2.1 Performance Tests of Gas Engine Using LPG and N_2

The modified gas engine was initially tested and operated in order to develop the proper technique and appropriate operating procedure for gas application testing.

Preliminary test runs using the modified gas engine were done by charging a constant flowrate of LPG (H.V. = 26,000 Kcal/Nm^3). Varying amounts of N_2 gas were mixed to determine the minimum practical heating value (H.V.) required for smooth start-up and operation of the engine.

During the test run, the flowrate of LPG was set constant at 0.24 Nm^3/h. At this flowrate, the engine was observed to perform smooth revolution in accordance with its specifications. The flowrate of N_2 was then varied and changes in the engine performance were examined. The pressure of the

gas (P) at the carburetor was affected by the H.V. of the resulting gas mixture. A correlation between LPG ratio and P is shown in Figure 22. At high H.V., the pressure was almost zero, however, at a lower value (about 2,300 Kcal/Nm^3), the pressure increased to about 680 mm (H_2O). For this reason, it was decided that the gas should pass from the governor directly to the carburetor due to the limited capacity of the pressure gauge (up to 500 mm H_2O).

Table 3 shows the results of the test runs. The minimum practical heating value for this run was found to be 2,540 Kcal/Nm^3. Below this value, a decreasing voltage output was observed. During this test run, a 600 watt electric heater was used as load.

2.2 Application Tests Using LPG and Air

Tests using another gas (mixture of LPG and air) were carried out. Table 6 shows the results of the test runs. It was revealed that 1,200 Kcal/Nm^3 was minimum heating value for smooth operation with an output of 100 volts. It was considered that gas with an even lower heating value could be used if further adjustment can be made in the operation of the engine.

2.3 Application Tests with Gas Fuel Product from the Air Gasification of Cellulosic Wastes.

Preliminary experiments on the gas fuel products obtained from sawdust were done at previously established operating conditions as shown in Table 4.

Experimental runs were carried out by air gasification method without the use of an external heater because it was noted that the heat evolved during the process was sufficient for the gasification to be self sustaining.

Based on the data obtained from the tests done on the gas engine which was operated using LPG and air as fuel, the gas fuel products generated from sawdust were likewise tested on the same engine. To start the engine, 0.24 Nm^3/h of LPG was introduced before the gas fuel product generated from sawdust was applied. Slowly increasing the flow rate of gas product being tested and decreasing flowrate of LPG were done simultaneously until a steady state fuel gas intake of about 60 liters were attained; after which the LPG supply was totally cut-off. At this condition, the output voltage was recorded as 40-65 volts at a gas pressure of 1,000 mm H_2O above. The pressure gauge used which has a maximum range of 1,000 mm H_2O was not sufficient to register the actual pressure reading.

To further test the effectivity of gas produced from sawdust and other cellulosic wastes (coirdust and copra meal), appropriate operating conditions (such as feedrate of raw material), flowrate of air, and bed tem-

peratures) on the gasification were examined to produce a gas product which possesses a H.V. higher than 1,000 Kcal/Nm^3. Minor adjustments on the engine were also made. The pressure gauge was changed to a higher capacity of 3,000 mm H_2O. Instead of collecting the gas in gas bags, the exit gas was fed directly to the engine. An additional filter column containing silica gel was attached before the gas was allowed to pass into the gas engine for actual testing. This step was added to further eliminate soot and mist from the gas. The composition and heating values of the produced gas fuel obtained at appropriate operating conditions are shown in Table 7.

The gas fuel products generated from each of the waste materials were tested on the gas engine. Results of the engine tests on the produced gas from sawdust is shown in Table 8. It is noteworthy that the engine was started using the produced gas alone even without preliminary LPG application. The engine run continuously in good condition at an average flowrate of 70 L/min of fuel gas with an output of 100 volts, 50-52 Hz at a maximum pressure of 1,200 mm H_2O. The power generated from the gas engine was tested on a 600 watt electric heater with comparable performance.

The results of the engine tests on the gas product generated from the gasification of copra meal and coirdust were almost the same as the result obtained from sawdust. By and large, the results of the studies undertaken on the application of gas product generated from the gasification of cellulosic wastes have shown their suitability to fuel gas engine for power generation.

CONCLUSIONS AND RECOMMENDATIONS

A continuous fluidized bed reactor system was developed and used for the study on the gasification of cellulosic wastes. The study has shown that steam and air or a combination thereof were suitable as fluidizing gases for gasification of sawdust, coirdust, copra meal and cassava bagasse.

Application tests were done on the gas products obtained from the various materials using a gas engine developed for the purpose. Results showed that low calorie gas product with heating value as low as 1,000 Kcal/Nm^3 can be utilized to run a gas engine for generation of electricity. Although the technology of using fuel gases of high calorific value (5,000 Kcal/Nm^3 and above) to run a gas engine is widely established, no such studies have been done yet using low calorific value as low as 1,100 Kcal/Nm^3 for power generation of electricity.

Air gasification can be adapted with advantage over steam for the following reasons:

1. No external heater is needed since the reaction is exothermic. Heat evolved from the reaction of oxygen on char from the raw materials can compensate for the heat needed for gasification.

2. Operating conditions can be conveniently controlled with ease.

3. Gas product of low heating value can be obtained that is applicable for gas engine operation to generate electricity.

R & D on a pilot plant scale is deemed necessary to gather engineering data and relevant information for evaluating the adaptability and suitability to local conditions.

Local adaptation and application of such novel finding would not only help solve waste disposal problems but would contribute to the socio-economic upliftment of our people in the rural areas and eventually help our country attain self-reliance in its energy needs.

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Table 1. Properties of raw materials used for gasification studies

Sample / Analyses	Sawdust A	Sawdust B	Sawdust C	Cassava bagasse	Copra meal	Coir dust	Coal
Proximate analysis (wt. %)							
Moisture	17.0	38.1	7.8	13.9	8.4	14.8	4.7
V.C.M.	72.7	55.4	79.6	74.1	73.9	60.1	45.9
Ash	1.9	0.5	0.7	2.6	5.1	8.4	10.2
F.C.	8.4	6.0	11.9	9.5	12.6	16.7	39.2
Ultimate analysis (wt. %)							
C	50.00			44.80	48.80	51.50	68.20
H	6.77			6.81	4.71	5.81	5.70
O	42.70			45.80	40.70	34.30	14.80
Ash	0.51			2.58	5.14	8.34	10.20
Heating value (cal/g)	4,810	3,270	4,370	4,230	4,730	4,090	---
Bulk density (g/mL)	0.24	0.63	0.29	0.35	0.57	0.10	---

Table 2. Properties of fluidizing sand employed.

Chemical composition	Toyoura sand (Silica sand)	Soma sand (Silica sand)	Olivine sand (Dunite)
SiO_2, %	92.6	97	40 - 44
Fe_2O_3, %	0.7		0.16-1.70
Al_2O_3, %	3.6		0.50-0.90
CaO, %	0.5	3	0.20-0.50
MgO, %	0.1	under	46-49
Ignition Loss, %	0.4		0.50-1.5
Bulk Density, g/mL	2.22	1.47	1.98

Table 3. Results obtained from steam gasification at ideal operating conditions.

Sample	Feedrate (kg/h)	Bed Temp. (°C)	Heating Value (Kcal/Nm^3)
Sawdust – A	1.0	900	3,480
Copra meal	1.4	800	3,650
Coir dust	0.6	850	2,850
Cassava bagasse	1.6	800	3,260

Table 4. Ideal conditions on air gasification.

Sample	Feedrate (kg/h)	Bed Temp. (°C)	Air (cm/sec)	Heating Value (kcal/Nm^3)
Sawdust – C	2.2	770	9.8	1,190
Copra meal	7.2	870	14.3	1,920
Cassava bagasse	7.1	790	13.0	1,725

Table 5. Results and data on the preliminary test run of gas engine with LPG and N_2 mixture. (Q_{LPG} = 0.24 Nm^3/h with electric heater, 600 watts load)

Expt. No.	Q_{N_2} (Nm^3/h)	P (mm H_2O)	V (volts)	H (hertz)	HV (kcal/Nm^3)	LPG Ratio
1	–	0	105	53.60	26,000	1.00
2	0.36	65	102	53.10	10,400	0.40
3	0.74	172	101	52.75	6,370	0.24
4	0.98	245	101	52.50	5,120	0.19
5	1.38	380	100	52.52	3,850	0.15
6	1.56	430	100	52.10	3,470	0.13
7	1.89	540	100	51.50	2,930	0.11
8	2.22	580	100	51.00	2,540	0.10
9	2.46	620	98	50.50	2,310	0.09
10	2.53	650	95	50.00	2,250	0.09

Table 6. Results and data on the test run of gas engine with LPG and air mixture. (Q_{LPG}=0.15 Nm^3/h with electric heater, 600 watts load)

Expt. No.	Q_{Air} (Nm^3/h)	P (mm H_2O)	V (volts)	H (hertz)	HV (kcal/Nm^3)	LPG Ratio	RPPM
1	0.06	20	105	53	18,460	0.71	3820
2	0.96	100	105	53	3,510	0.13	3718
3	1.32	300	105	53	2,650	0.10	3460
4	1.68	600	105	53	2,130	0.08	3313
5	1.98	900	105	53	1,830	0.07	3879
6	2.28	1,200	105	53	1,610	0.06	3578
7	2.70	1,200	105	53	1,370	0.05	3868
8*	2.52	1,200	100	53	1,170	0.04	3800

*LPG 0.12 Nm^3/h

Table 7. Composition of gas produced from cellulosic wastes.

Materials	Bed Temp. (°C)	F (kg/hr)	GAS CONCENTRATION (%)									HV (kcal/Nm^3)
			H_2	O_2	N_2	CH_4	CO_2	C_2H_4	C_2H_6	CO	C_3H_8	
Sawdust	750	2.70	5.59	0.04	53.9	2.97	11.0	1.01	0.09	13.4	0.23	1,090
	760	2.70	5.55	0.29	53.7	2.95	11.0	0.92	0.24	13.3	0.26	1,080
	750	2.90	5.37	0.41	69.5	2.80	4.0	1.20	0.18	12.0	0.05	1,010
	812	3.00	6.56	1.11	53.2	4.04	12.9	1.93	0.22	16.8	0.11	1,440
Coirdust	850	4.30	8.75	0.59	54.2	3.09	15.9	1.18	0.17	14.0	0.18	1,240
	850	4.30	10.84	0.14	52.0	3.08	15.5	1.11	0.17	14.8	0.17	1,310
	850	4.30	10.78	0.28	52.2	3.04	15.4	1.12	0.17	14.7	0.16	1,300
	848	4.30	8.84	0.55	54.0	3.14	16.0	1.15	0.17	14.0	0.18	1,240
	864	2.30	8.87	0.33	57.5	2.31	14.4	0.96	0.09	13.7	0.07	1,090
	768	2.10	7.08	0.33	61.3	3.40	7.5	1.34	0.23	13.2	0.15	1,210
Copra meal	795	2.90	6.91	0.43	63.3	2.37	11.0	2.37	0.20	8.65	0.38	1,200
	702	3.50	6.63	1.39	63.1	2.22	11.7	1.74	0.35	7.57	0.67	1,140
	764	3.80	8.78	0.45	56.6	3.23	12.1	2.63	0.41	9.59	0.80	1,530
	664	4.60	6.66	0.55	59.5	2.59	13.1	1.64	0.47	10.20	0.04	1,020
	700	5.30	6.51	4.62	59.3	2.51	9.7	1.73	0.43	8.07	0.73	1,210

Table 8. Application test of gas fuel product from sawdust.
(T_b = 750°C. F - 2.7 kg/h, HV_{gas} = 1,090 kcal/Nm^3)

Flowrate of fuel gas (L/min)	P (mm H_2O)	V (volts)	H (hertz)	Remarks
*35	500	10		
50	700	60		
50	800	70		
59	1,000	75		
60	1,000	85	45.5	Loaded with 300 watts elec. heater
70	1,200	95		
70	1,200	98		
70	1,200	100	51.5	600 watts
72	1,200	100	52.0	elec. heater attached during operation

*Flowrate to start the engine without LPG.

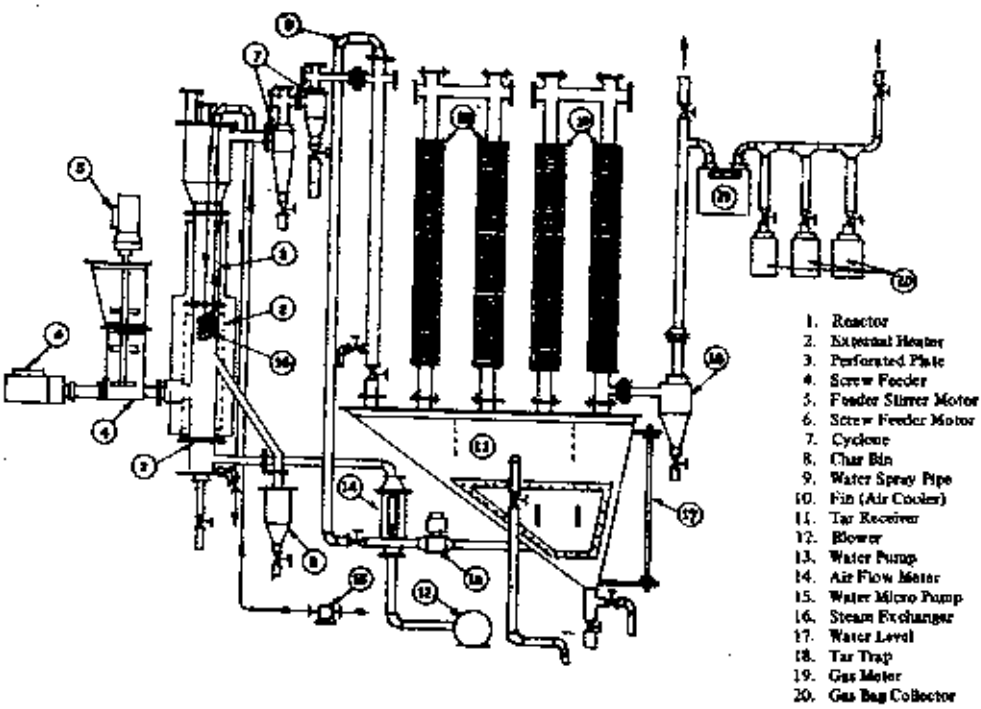

Fig. 1. Bench scale gasification apparatus.

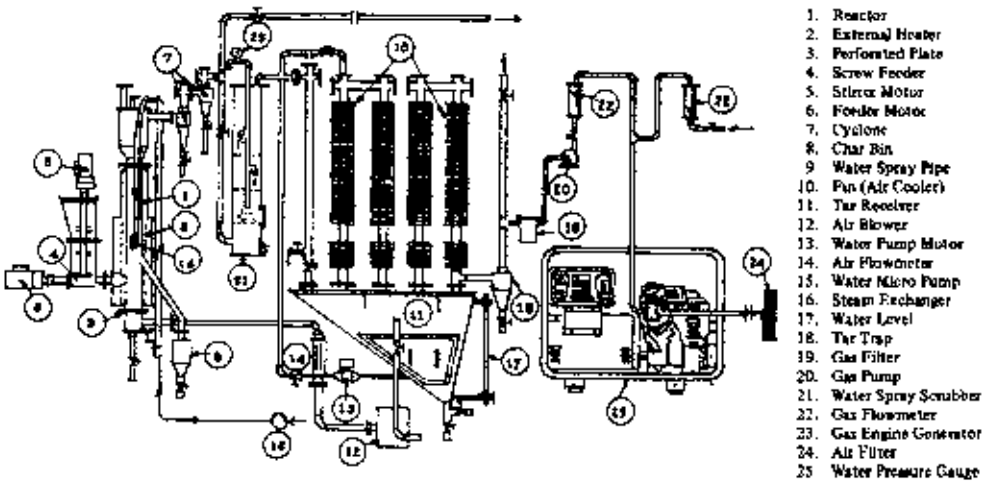

Fig. 2. Schematic diagram of continuous fluidized bed method gasification apparatus.

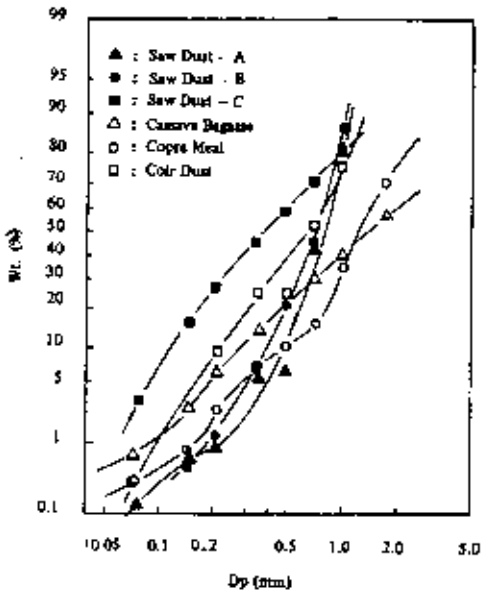

Fig. 3. Screen analysis of raw materials.

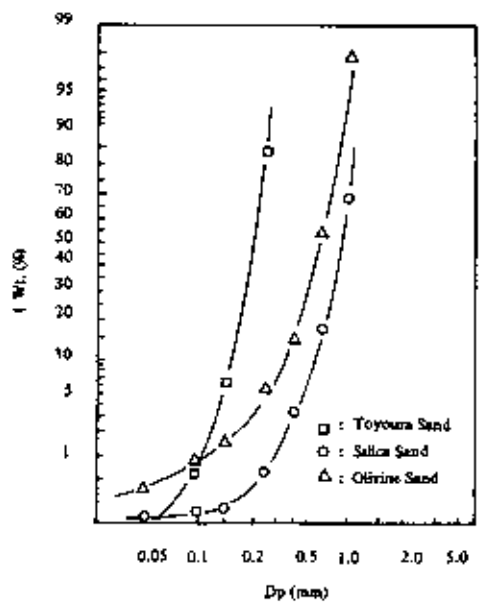

Fig. 4. Screen analysis of sand.

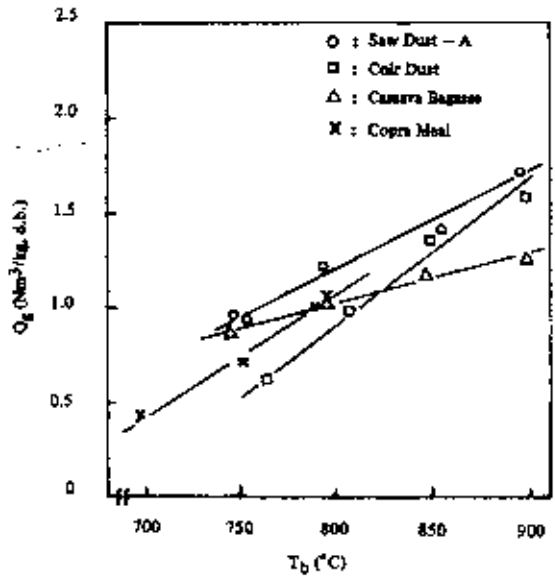

Fig. 5. Effect of bed temperature (T_b) on volume of gas product (Q_g) in steam gasification.

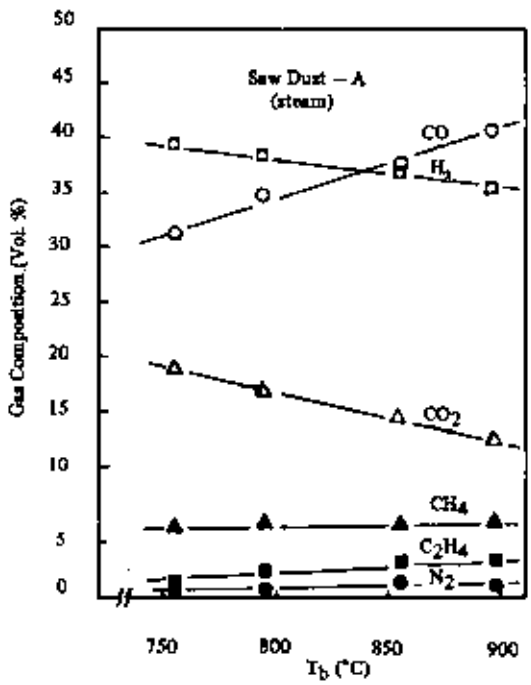

Fig. 6. Correlation between gas composition (vol. %) and bed temperature (T_b).

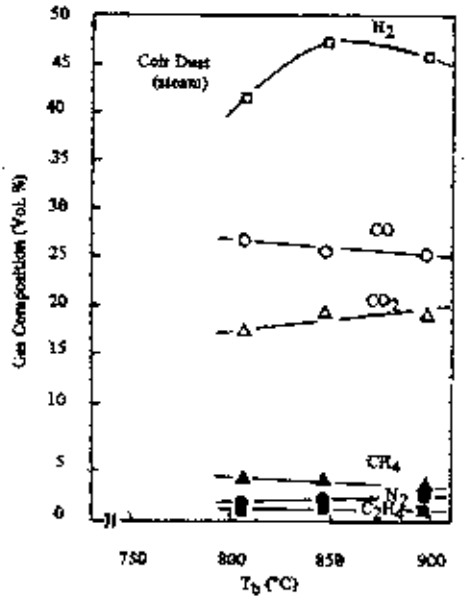

Fig. 7. Correlation between gas composition (vol. %) and bed temperature (T_b).

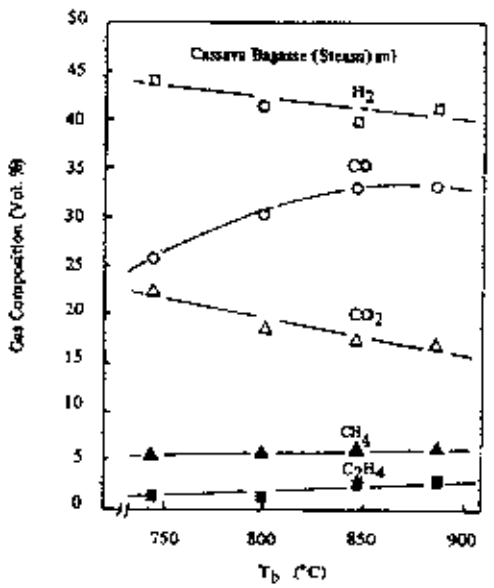

Fig. 8. Correlation between gas composition (vol. %) and bed temperature (T_b).

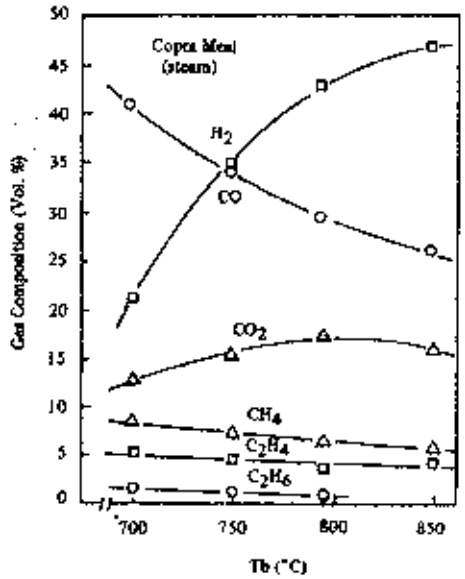

Fig. 9. Correlation between gas composition (vol. %) and bed temperature (T_b).

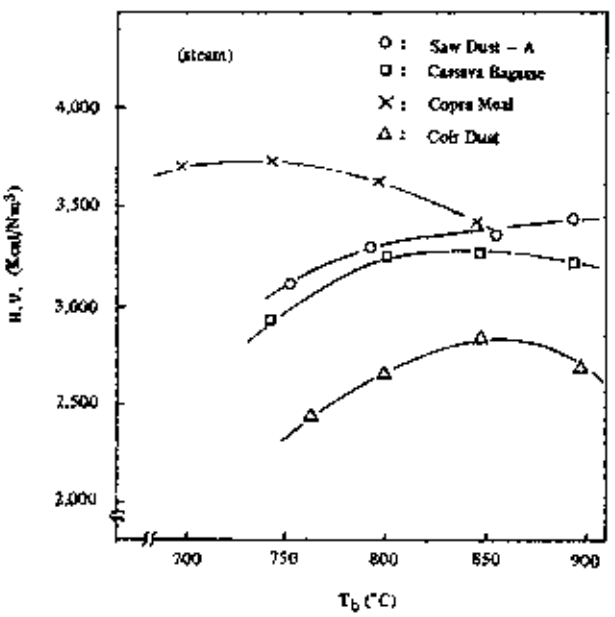

Fig. 10. Effect of bed temperature (T_b) on the heating value (H.V.) of gas product.

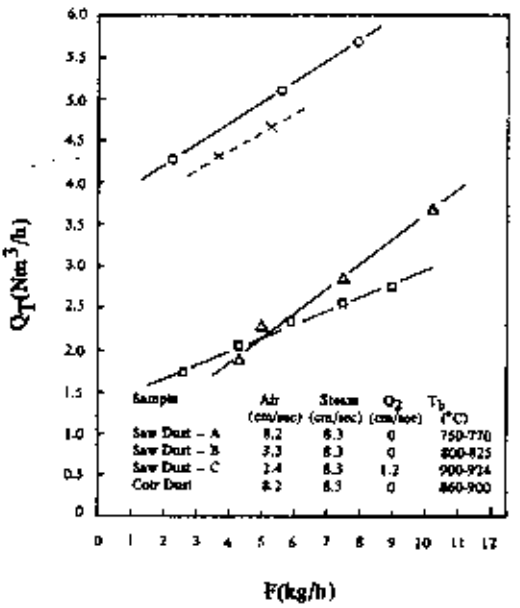

Fig. 11. Effect of feedrate (F) on gas yield (Q_T) on air and steam gasification.

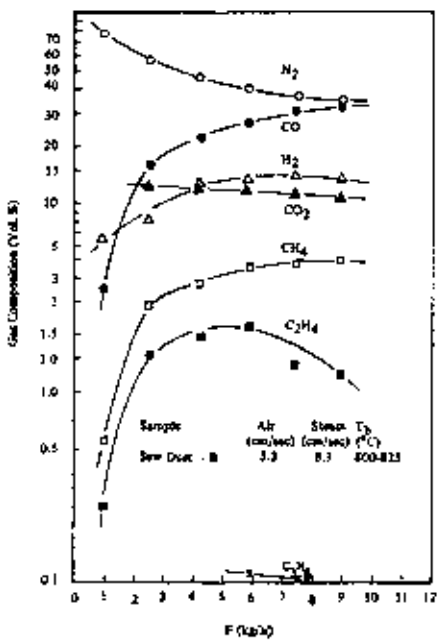

Fig. 12. Effect of feedrate (F) on the gas composition.

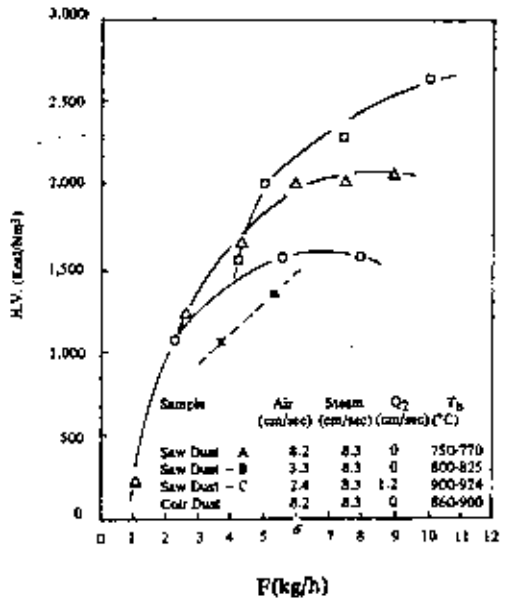

Fig. 13. Effect of feedrate (F) on the heating value (HV) of gas product.

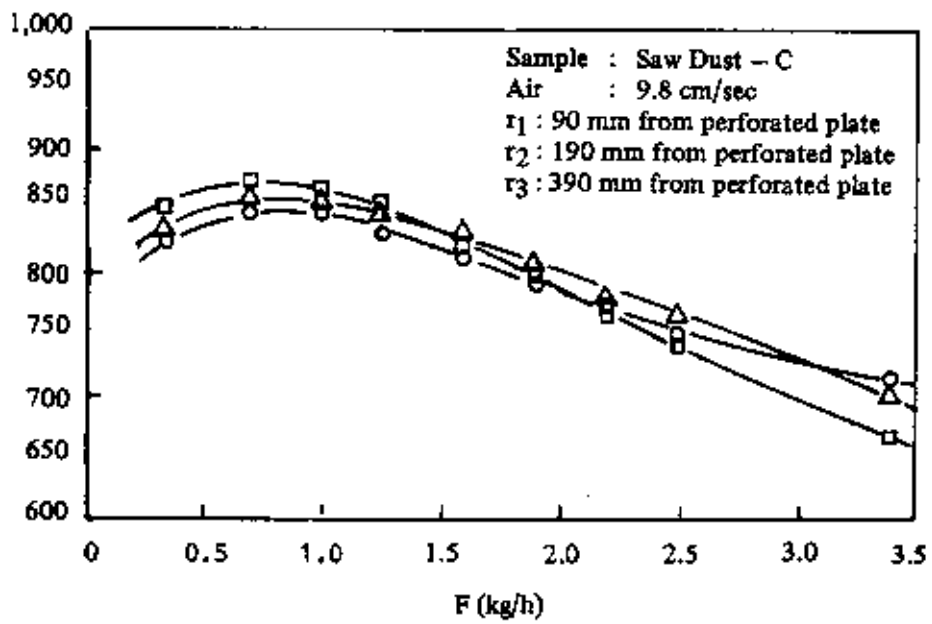

Figure 14. Correlation between feed rate (F) and bed temperature (T_b).

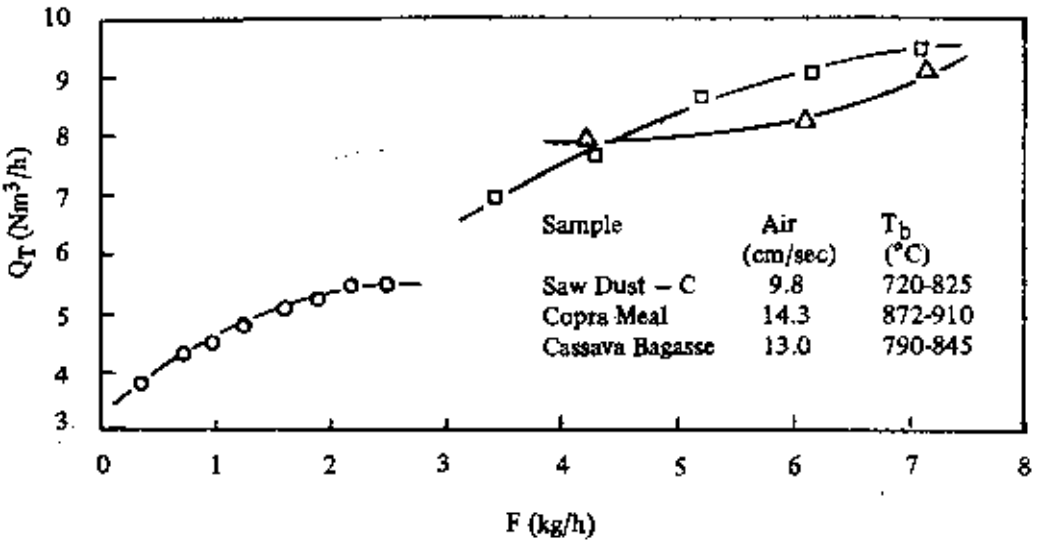

Fig. 15. Correlation between volume of gas (Q_T) and feed rate (F).

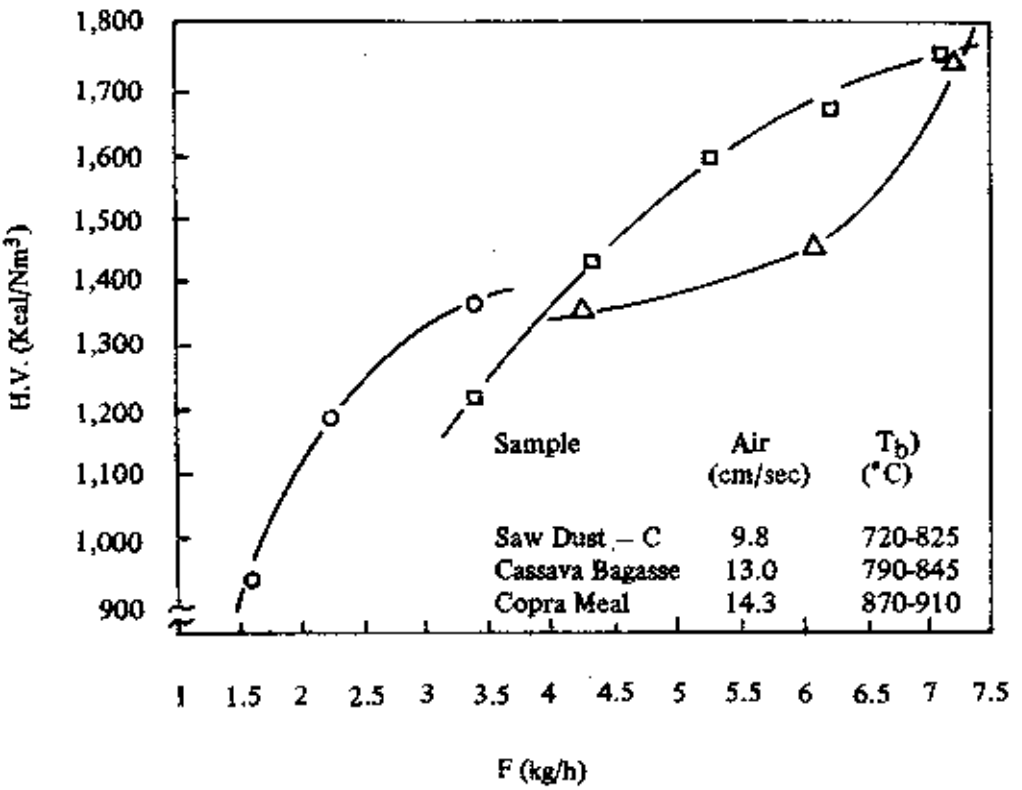

Fig. 16. Effect of feed rate (F) on the heating value (H.V.) of gas product.

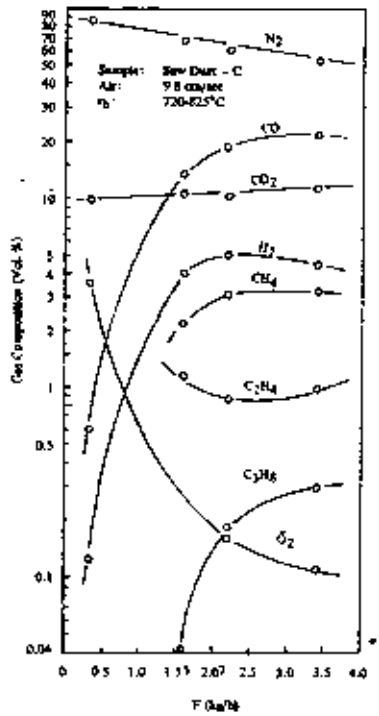

Fig. 17. Effect of feed rate (F) on the gas composition.

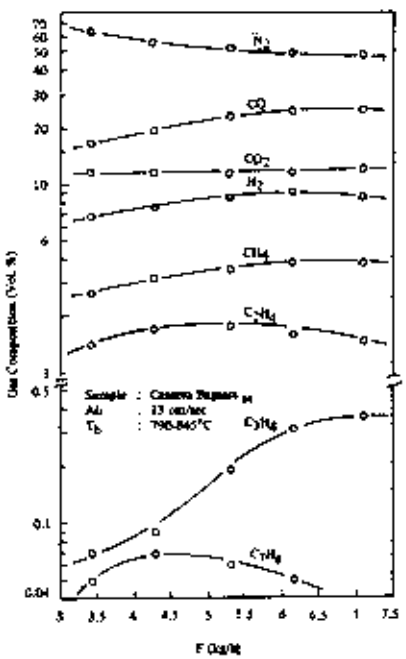

Fig. 18. Effect of feed rate (F) on the gas composition.

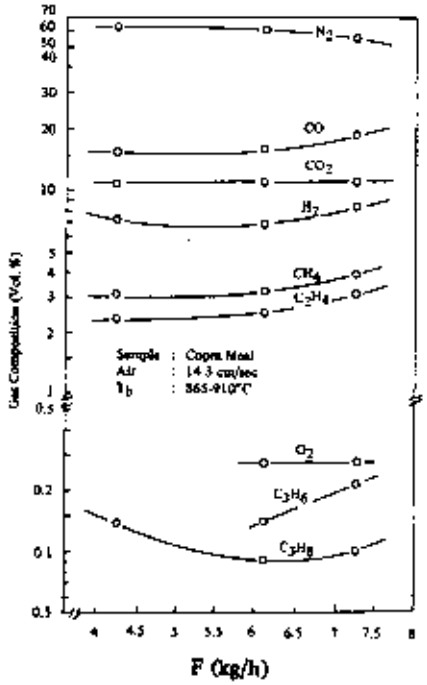

Fig. 19. Effect of feed rate (F) on the gas composition.

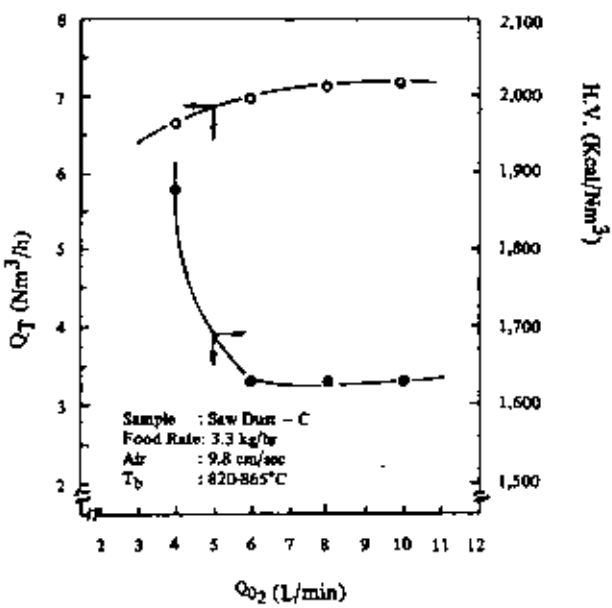

Fig. 20. Effect of oxygen (Q_{O2}) on the volume (Q_T) and heating value (H.V.) of gas product.

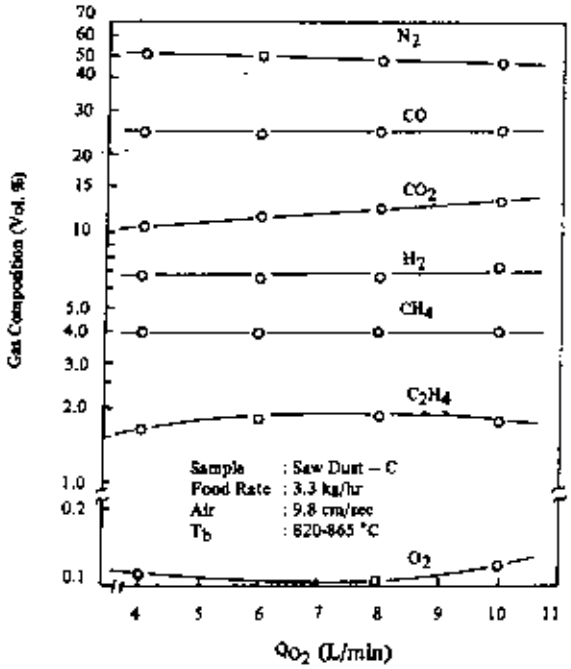

Fig. 21. Effect of oxygen (Q_{O_2}) on the gas composition.

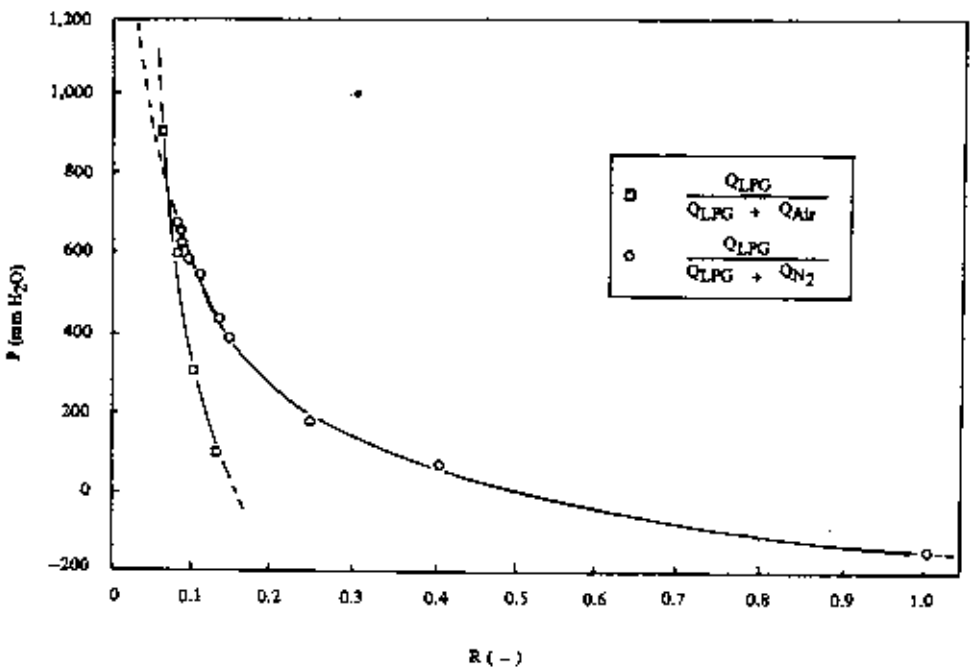

Fig. 22. Correlation between LPG ratio (R) and gas pressure (P).

SCREENING OF SOME LOCAL PLANTS FOR ANTIFERTILITY ACTIVITY IN FEMALE MICE

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ABSTRACT

Using a modified screening method of Hall *et al* for antifertility activity in female mice, extracts and/or juices of the following plants were tested for antifertility activity: *Vernonia cinerea* (Linn.) Less. ("tagulinai"), *Tinospora rumphii* Boerl ("makabuhay"), *Moringa oleifera* Lam. ("malunggay"), *Artemisia vulgaris* Linn. ("damong maria"), *Dioscorea esculenta* (Lour.) Burkill ("tugi"), *Momordica charantia* Linn. ("ampalaya") and *Averrhoa bilimbi* Linn. ("kamias"). The following extracts/juices gave promising results (50% or more reduction in fertility v.s. the control group): petroleum ether extract of *V. cinerea* dried whole plant at 1 g/kg dose, methanol extract of *T. rumphii* dried stems at 0.9 g/kg dose, 80% ethanol extract of *M. charantia* dried fruits at 0.352 g/kg dose, *A. bilimbi* fruit juice at 14 mL/kg dose and the ethanol extract of its dried fruit at 1 g/kg dose.

INTRODUCTION

The review article entitled "Philippine Local Plants as Sources of Antifertility Agents" prepared by the senior author (1984) includes a list of plants found to be active in animals as antifertility agents. Most of them are common plants such as *Aloe vera* or "sabila" leaves; *Ananas comosus* or pineapple unripe fruit and leaves; *Anona squamosa* or "atis" seeds; *Areca catechu* or "bunga" fruit, *Carica papaya* or "papaya" unripe fruit pulp; *Catharanthus roseus* or "chichirica" leaves; *Daucus carota* or carrot seeds; *Hibiscus rosasinensis* or "gumamela" flowers, leaves, stems and bark; *Leucaena leucocephala* or "ipil-ipil" leaves, *Momordica charantia* or "ampalaya" fruit; *Phaseolus aureus* or "mongo" whole plant, *Piper nigrum* or "paminta" fruit, *Pisum sativum* or "chicharo" seeds and *Raphanus sativus* or "labanos" whole plant. The active principles from some of these plants have been isolated and found to belong to different classes of compounds such as alkaloids, organic acids, phenolic compounds and sterols. These data clearly indicate the potential of our local plants as sources of antifertility drugs.

To date, there has been no report on the antifertility activity of plants here in the Philippines. This is one of the reasons for our undertaking a project study of local plants as possible sources of antifertility drugs. Based on our literature research, we tried several methods of testing until we came up with a method that gave consistent and reproducible results.

MATERIALS AND METHODS

A. Collection and processing of plant materials

Vernonia cinerea (L.) Less. or "tagulinai" was collected from open waste places in Manila and Quezon City. The roots were washed in running water and the plants were air-dried and ground in a Wiley Mill.

Tinospora rumphii Boerl. or "makabuhay" stems were collected from Cavite along the riverbanks. The stems were cut crosswise into thin slices, air-dried and ground.

Moringa oleifera Lam. or "malunggay" young shoots were bought from Quiapo market. The leaves were separated, air-dried and ground.

Artemisia vulgaris L. or "damong maria" aereal parts were collected from the backyards of San Jose, Batangas. The leaves were separated, air-dried and ground.

Dioscorea esculenta (Lour.) Burkill or "tugi" tubers obtained from Lopez, Quezon were pared, cut into thin slices and dried in an oven at about 60°C and ground.

Momordica charantia Linn. or "ampalaya" fruits were bought from Quiapo market. The seeds and seed pulp were removed and the remaining fleshy portion was cut crosswise into thin slices and air-dried.

Averrhoa bilimbi L. or "kamias" fruit were collected from a tree in Sta. Ana, Manila. The calyx were removed and the fruit was sliced lengthwise in thin slices and dried in an oven at 40-60°C. The dried fruit was ground and kept in a desiccator prior to extraction.

B. Preparation of the extracts/juices

1. *V. cinerea*

(a) Methanol extract

The dried powdered plant was extracted with anhydrous methanol A. R. in a Soxhlet extractor for about two (2) weeks. The extracts were filtered, concentrated under vacuum and evaporated to dryness over a water bath.

(b) Petroleum ether extract

The dried powdered whole plant was extracted with petroleum ether A.R. in a Soxhlet extractor for 4-5 days. The extracts were filtered, concentrated under vacuum and evaporated to dryness over a water bath.

2. *T. rumphii*

The dried powdered stems were extracted with methanol A.R. in a Soxhlet extractor for about three (3) weeks. The extracts were filtered, concentrated under vacuum and evaporated to dryness over a water bath.

3. *M. oleifera*

The ground dried leaves were extracted with 80% ethanol by percolation until the extract was light in color. The combined extracts were concentrated under vacuum and evaporated to dryness over a water bath.

4. *A. vulgaris*

The ground dried leaves were extracted with 95% ethanol in a Soxhlet extractor after previous defatting with petroleum ether (35-60°C b.p.). The alcoholic extracts were combined, concentrated under vacuum and evaporated to dryness over a water bath.

5. *D. esculenta*

The ground dried tubers were extracted in a Soxhlet extractor with 95% ethanol for about two (2) weeks. The extracts were concentrated and evaporated to dryness.

6. *M. charantia*

The ground dried fruit was extracted with petroleum ether by percolation until the extract was light in color. The marc was then re-extracted with 80% ethanol also by percolation. The alcoholic extract was concentrated under vacuum and evaporated to dryness.

7. *Averrhoa bilimbi*

(a) Juice

The calyx of the fresh fruit was cut out and the remaining portion was comminuted, pounded and the juice squeezed off through muslin cloth.

(b) Extracts of the dried fruit

The ground dried fruit was extracted successively in a Soxhlet extractor with petroleum ether, b.p. 35-60°C, ether anhydrous and 95% ethanol. The extracts were concentrated under vacuum and evaporated to dryness.

C. Antifertility Testing

The methods used were adopted from the Female Mouse Antifertility Screen of Hall, I.C. *et al*, (1974) with some modifications. Methods A and B were outlined as follows:

Method A

Grouping, numbering and weighing of the female mice (6 mice/grp)

Drug administration for 10 days

Weighing and mating of the mice (1 male/2 females)

Drug administration to the females for 18 days while mating

Sacrifice and autopsy of the females noting the number and appearance (normal or abnormal) of the fetuses.

Method B

Grouping, numbering and weighing of the female mice (6 mice/grp)

Drug administration for 10 days

Weighing and mating of the mice (1 male/2 females)

Drug administration to the females for 10 days while mating

Stop drug administration and withdraw males for 8 days

Sacrifice and autopsy of the females noting the number and appearance (normal or abnormal) of the fetuses.

The drug was suspended in 10% Tween 80 in distilled water and administered orally by use of a polyethylene tubing attached to a hypodermic syringe with a gauge 21 needle. Grouping was made as follows:

Group I	*Group II*	*Group III, IV, etc.*
Negative Control	Positive Control	Test Groups
Solvent 10% Tween 80 in distilled water or plain distilled water	Enovid (5 mg tablets) powdered and suspended in the solvent	Extract* suspended in the solvent
Dose: 2 mL/mouse	3 mg/kg norethynodrel	1 g/kg crude extract or juice/extract from 5, 10 and 20 g fresh plant material

*If fruit juice is used, no solvent is required and tap or distilled water is used as negative control.

Method A was used to screen the extracts of *V. cinerea* and *T. rumphii*. Method B was used to screen the juices/extracts of *A. bilimbi*, *D. esculenta*, *M. charantia*, *A. vulgaris* and *M. oleifera*.

A maximum dose of 1·2 g extract was used. This weight of extract was usually obtained from 10 g crude dried drug which in turn was obtained from 20 g fresh plant material. Thus the extract or juice obtained from 20 g fresh plant material was used as the highest dose.

RESULTS AND DISCUSSION

Results shown in Tables 1-7 are summarized in the table below:

	Plants	*Plant part used*	*Type of extract tested*	*Dose*	*% reduction in fertility over the control**
1.	*Vernonia cinerea*	whole plant	MeOH extract	1g/kg	7.5
			Petroleum ether extract	1g/kg	60.0
2.	*Tinospora rumphii*	stems	MeOH extract	0.9g/kg	55.0
3.	*Moringa oleifera*	leaves	80% EtOH extract	1g/kg	3.33
4.	*Artemisia vulgaris*	leaves	95% EtOH extract	1g/kg	3.33
5.	*Dioscorea esculenta*	tubers	95% EtOH extract	0.854g/kg	33.33
6.	*Momordica charantia*	fruit	80% EtOH extract	0.352g/kg	58.23
7.	*Averrhoa bilimbi*	fresh fruits	juice	14mL/kg	58.23
		dried fruits	95% EtOH extract	1 g/kg	66.67

*50% or more reduction in fertility is considered promising.

The following extracts /juices are therefore considered promising and could be utilized for further studies: petroleum ether extract of *V. cinerea* dried whole plant, methanol extract of *T. rumphii* dried stems, 80% ethanol extract of *M. charantia* dried fruit and *A. bilimbi* fruit juice and ethanol extract of the dried fruit. Less toxic extracts

or a detoxified methanol extract of *T. rumphii* could be utilized instead of the methanol extract since at the effective dose (0.9 g/kg), one third of the experimental mice died.

SUMMARY AND CONCLUSIONS

1. Using a modified method of Hall *et al* (1974) for antifertility activity in female mice, the following extracts/juices were found promising (eliciting 50% or more decrease in fertility): petroleum ether extract of *V. cinerea* at 1 g/kg dose, methanol extract of *T. rumphii* at 0.9 g/kg dose, 80% ethanol extract of *M. charantia* at 0.352 g/kg dose, fruit juice of *A. bilimbi* at 14mL/kg dose and the ethanol extract of *A. bilimbi* at 1 g/kg dose. In the case of *T. rumphii*, less toxic extracts other than methanol extract of the dried stems could be utilized.

2. On the other hand, the following preparations produced less than 50% reduction in fertility: methanol extract of *V. cinerea*, 80% ethanol extract of *M. oleifera* and 95% ethanol extract of *A. vulgaris* at 1 g/kg doses respectively and the 95% ethanol extract of *D. esculenta* at 0.854 g/kg dose.

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Table 1. Antifertility activity of the methanol extract of the whole plant of *V. cinerea*.

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	10% Tween 80 in distilled water	0.3mL/mouse	8	1	12
II.	Enovid 5 mg tablet	3mg/kg	8	8	100
III	*V. cinerea* MeOH extract	0.5g/kg	5	1	20
IV	– do –	1.0g/kg	5	1	20
V	– do –	1.5g/kg	8	2	25

Note: This was repeated and almost the same results were obtained.

Table 2. Antifertility activity of the petroleum ether extract of the whole plant of *V. cinerea*.

Grp.	Drug Administered	Dose	n	No. of Mice w/o fetuses	% Infertile
I	10% Tween 80 in distilled water	0.3mL/mouse	5	0	0
II	Enovid 5 mg tablet	3mg/kg	4	4	100
III	*V. cinerea* petroleum ether extract	1g/kg	5	3	60

Table 3. Antifertility activity of the methanol extracts of the stems of *T. rumphii*.

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	10% Tween 80 in distilled water	0.3mL/mouse	5	1	20
II	Enovid 5 mg tablet	3mg/kg	6	6	100
III	*T. rumphii* MeOH ext.	0.1g/kg	5	0	0
IV	– do –	0.2g/kg	5	1	20
V	– do –	0.3g/kg	6	2	33
VI	– do –	0.6g/kg	6	3	50
VII	– do –	0.9g/kg	4	3	75

Note: The above results were consolidated from two separate experiments.

Table 4. Antifertility activity of the ethanol extract of the leaves of *M. oleifera*.

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	Distilled water	0.2mL/ mouse	6	1	17
II	Enovid 5mg tablet	3 mgs/kg	6	6	100
III	*M. oleifera* 80% ETOH extract	0.5mg/kg	6	0	0
IV	-- do --	1.0g/kg	5	1	20
V	-- do --	2.0g/kg	5	1	20

Table 5. Antifertility activity of the ethanol extract of the leaves of *A. vulgaris*

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	10% Tween 80 in distilled water	-0.2mL/ mouse	6	1	17
II	Enovid 5mg tablet	3mgs/kg	4	4	100
III	*A. vulgaris* ETOH extract	0.25g/kg	6	1	17
IV	-- do --	0.5g/kg	5	2	40
V	-- do --	1.0g/kg	4	2	50

Table 6. Antifertility activity of the ethanol extract of the dried tubers of *D. esculenta*.

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	10% Tween 80 in distilled water	0.2mL/ mouse	6	1	17
II	Enovid 5mg tablet	3mg/kg	5	5	100
III	*D. esculenta* ETOH extract	0.213g/kg	6	1	17
IV	-- do --	0.427g/kg	6	2	30
V	-- do --	0.854g/kg	4	2	50

Table 7. Antifertility activity of the ethanol extract of the dried fruit of *M. charantia*.

Grp.	Drug Administered	Dose	n	No. of mice w/o fetuses	% Infertile
I	Distilled water	0.2mL/ mouse	6	1	17
II	Enovid 5mg tablet	3mg/kg	6	6	100
III	*M. charantia* ETOH extract	0.088g/kg	5	2	40
IV	– do –	0.176g/kg	6	3	50
V	– do –	0.352g/kg	4	3	75

MUTAGENICITY, CLASTOGENICITY AND ANTIMUTAGENICITY OF MEDICINAL PLANT TABLETS PRODUCED BY THE NSTA PILOT PLANT III. *LAGUNDI* TABLETS

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ABSTRACT

"Lagundi" tablets from the NSTA pilot plant did not show DNA-damaging capacity. They were not mutagenic before and after metabolic activation nor did they show clastogenic or chromosome-breaking effects.

However, "lagundi" tablets reduced the micronucleated polychromatic erythrocytes induced by dimethylnitrosamine, N-nitrosopyrrolidine and tetracycline, indicating the antimutagenic activity of the tablets.

INTRODUCTION

Decoctions of leaves of "lagundi" (*Vitex negundo* L.) are used for fever and cough (Philippine National Formulary, 1980).

The NSTA Pilot Plant produced "lagundi" tablets from dried leaves, using starch and magnesium stearate as fillers.

It is imperative to know if these "lagundi" tablets are genotoxic and if not, if they are antimutagenic.

MATERIALS AND METHODS

The Rec assay (Kada *et al*, 1980) was utilized for determining direct DNA damaging potential. To study mutagenicity potential before metabolic activation, a microbial test was used (Ames, 1975). To determine mutagenicity potential after metabolic activation, the host-mediated assay (Moriya, 1980) was employed. To determine the clastogenicity potential or chromosome-breaking potential, the micronucleus test was employed (Schmid, 1975). The same test was employed in studying antimutagenic effects against tetracycline, dimethylnitrosamine and N-nitrosopyrrolidine.

RESULTS AND DISCUSSION

The results of the Rec assay are shown in Table 1. "Lagundi" tablets did not produce zones of inhibition and therefore they do not possess direct DNA damaging capacity.

The results of the Ames test are shown in Tables 2 and 3. *Salmonella typhimurium* tester strains TA 97 and TA 100 were used. Both strains do not possess the excision repair system and they contain the R plasmid which makes them sensitive towards systems that are mutagenic before metabolic activation. Both are sensitive towards frame-shift mutagens. The number of revertants per plate did not differ significantly from the control. Thus, "lagundi" tablets are not mutagenic before metabolic activation.

Data for mutagenicity potential after metabolic activation are shown in Table 4. The mutation frequency due to "lagundi" tablets is at the same range as the control indicating that these tablets are not metabolized to mutagens and no metabolite from the tablets reacted with DNA.

The results of the micronucleus test are shown in Table 5. Data show that "lagundi" tablets are not genotoxic to bone marrow cells. They are not clastogenic and they do not possess chromosome-breaking properties.

Table 6 shows that "lagundi" tablets reduce the genotoxicity of dimethylnitrosamine which is not only a mutagen but also a carcinogen (Fishbein, 1978). The number of micronucleated polychromatic erythrocytes was greatly reduced to the level of the control indicating antimutagenic effects of "lagundi" tablets.

Table 7 shows that "lagundi" tablets are antimutagenic against N-nitrosopyrrolidine which is also a mutacarcinogen (Lijinsky, 1976). These tablets greatly reduced the formation of micronucleated polychromatic erythrocytes in bone marrow cells.

Table 8 shows the reduction of genotoxicity of tetracycline by "lagundi" tablets. Tetracycline is not only a mutagen but also a teratogen (Sylianco and Blanco, 1984).

Both dimethylnitrosamine and N-nitrosopyrrolidine are metabolized to alkylating agents of DNA (Lijinsky, 1976). This is the mechanism that induces genotoxicity. The "lagundi" tablets therefore, reduce the tendency of these N-nitroso compounds to alkylate DNA.

Tetracycline is genotoxic not only to somatic cells but also to germ cells (Sylianco and Blanco, 1984). Its genotoxicity is a consequence of intercalation-interactions with base-pairs in DNA. "Lagundi" tablets, by reducing its genotoxicity must have reduced the intercalating capacity of tetracycline with DNA.

SUMMARY AND CONCLUSION

1. The "lagundi" tablets from NSTA pilot plant do not possess direct DNA-damaging capacity. They do not exhibit mutagenic activity before and after metabolic activation and are not genotoxic to bone marrow cells.

2. The "lagundi" tablets exhibit antimutagenic effects as evidenced by the reduced formation of micronucleated polychromatic erythrocytes induced by the three mutagens dimethylnitrosamine, N-nitrosopyrrolidine and tetracycline.

ACKNOWLEDGEMENT

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Table 1. DNA-damaging potential of "lagundi" tablets.

	Zones of inhibition (mm)	
	Rec^+	Rec^-
Positive control*	22.4 ± 1.5	33.1 ± 1.3
Negative control**	0	0
Placebo tablet***	0	0
"Lagundi" tablet		
30 mg/75 mL	0	0
30 mg/37.5 mL	0	0
30 mg/1.38 mL	0	0

* –4 nitroquinoline oxide
**distilled water
***contains starch and magnesium stearate

Table 2. Mutagenicity potential before metabolic activation of "lagundi" tablets using *Salmonella typhimurium* TA 100.

	Revertants per plate*
Positive control**	Too numerous to count
Negative control***	20.20 ± 1.47
Placebo tablet	20.84 ± 2.31
Control	21.52 ± 1.87
"Lagundi" tablet	
30 mg/75 mL	20.80 ± 2.22
30 mg/37.5 mL	20.32 ± 1.65
30 mg/18.8 mL	20.16 ± 2.45

**Salmonella typhimurium* TA 100
**ethylmethanesulfonate
***distilled water

Table 3. Mutagenicity potential before metabolic activation of "lagundi" tablets using *S. typhimurium* TA 97.

	Revertants per plate*
Positive control**	Too numerous to count
Negative control***	21.12 ± 2.27
Placebo tablet	22.04 ± 1.94
"Lagundi" tablet	
30 mg/75 mL	19.92 ± 1.29
30 mg/37.5 mL	20.88 ± 1.99
30 mg/18.8 mL	22.28 ± 2.34

**Salmonella typhimurium* TA 97
**Methylmethanesulfonate
***Distilled water

Table 4. Mutagenicity potential after metabolic activation of "lagundi" tablets using *S. typhimurium* G 46.

	Mutation frequency*
Positive control**	7.26 ± 1.14
Negative control***	1.54 ± 0.76
Placebo tablet	1.60 ± 0.28
"Lagundi" tablet	
20 mg/kg body wt	1.44 ± 0.71
40 mg/kg body wt	1.42 ± 0.69
10 mg/kg body wt	1.40 ± 0.47

**Salmonella typhimurium* G 46
**dimethylnitrosamine
***distilled water

Table 5. Clastogenicity potential of "lagundi" tablets.

	No. of micronucleated polychromatic erythrocytes per thousand
Positive control*	12.45 ± 1.23
Negative control**	1.83 ± 0.50
Placebo tablet	1.47 ± 0.91
"Lagundi" tablet	
10 mg/kg body wt	1.13 ± 0.56
20 mg/kg body wt	1.13 ± 0.69
40 mg/kg body wt	1.40 ± 0.64

*dimethylnitrosamine
**distilled water

Table 6. Antimutagenic effects of "lagundi" tablets against dimethylnitrosamine.

	No. of micronucleated polychromatic erythrocytes per thousand
Dimethylnitrosamine, 15 mg/kg	12.45 ± 1.23
DMN plus "lagundi" tablet	
10 mg/kg	1.27 ± 0.50
20 mg/kg	1.47 ± 0.19
40 mg/kg	1.40 ± 0.43
Control	1.76 ± 0.89

Table 7. Antimutagenic effects of "lagundi" tablets against N-nitrosopyrrolidine.

	No. of micronucleated polychromatic erythrocytes per thousand
N-nitrosopyrrolidine (N-NP), 325 mg/kg	6.83 ± 1.48
N-NP plus "lagundi" tablet	
10 mg/kg	1.47 ± 0.51
20 mg/kg	1.93 ± 0.28
40 mg/kg	1.53 ± 0.80
Control	1.55 ± 0.97

Table 8. Antimutagenic effects of "lagundi" tablets against Tetracycline.

	No. of micronucleated polychromatic erythrocytes per thousand
Tetracycline, 55 mg/kg	13.50 ± 0.63
Tet plus "lagundi" tablet	
10 mg/kg	1.20 ± 0.60
20 mg/kg	1.86 ± 0.51
40 mg/kg	1.27 ± 0.55
Control	1.43 ± 1.03

GONIOPHLEBIUM OF THE PHILIPPINES (POLYPODIACEAE)

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ABSTRACT

Polypodium mengtzeense Christ is transferred to *Goniophlebium*. *Goniophlebium tomentellum* (C.Chr.) Copel. and *Goniophlebium integrum* Copel are regarded synonymous to *Goniophlebium subauriculatum* (Bl.) Presl. A key to 7 Philippine species of *Goniophlebium* is presented, followed by their respective taxonomical history and a description.

INTRODUCTION

This paper is a taxonomic revision of the *Goniophlebium* species occurring in the Philippines. It is part of a monographic study of *Goniophlebium* by the author, including full synonymy and descriptions of all species, which is being undertaken as part of the Polypodiaceae Project (Hennipman, 1986).

Goniophlebium as defined here includes the Polypodiaceae with goniophleboid venation i.e., "forking and anastomosing to form a series of costal areolae, each with one free-included veinlet excurrent from the base of the inferior main vein, beyond these marginal free veins or additional areolae with or without included free veinlets" (Copeland, 1960) and with spores with crestately folded perispore (Fig. 1).

MATERIALS AND METHODS

I have been collecting all *Goniophlebium* species reported from the Philippines, including the species named by Copeland from their type localities. A considerable number of plants of all species is now growing in my private garden in Manila for the observation of frond development and life cycles.

All relevant herbarium materials were studied from the following herbaria: BO, CAHP, L, P, PNH, SING and U. The types of the species described by Copeland were received on loan from MICH.

The living material allowed the selection of 115 different morphological characters, which were studied from at least 5 samples per species. The microscopical observations, measurements and drawings of details were made using a CHA-Olympus light microscope

with drawing attachment. Semi-permanent slides were prepared by embedding the object in glycerin jelly. Projection photographs of venation patterns were taken following the procedure described by Hennipman (1977). The descriptive terminology used follows that of Stearn (1983). Index values of scales and fronds indicate the relation between length and largest width.

Description of *Goniophlebium* in the Philippines

Herbs, epiphytic, epilithic or terrestrial, moderate or large-sized. *Rhizomes* long-creeping, 1.5-11 mm in diam., branched, round or dorsiventrally flattened, clothed with scales, green or glaucous, sclerenchyma strands present in specific numbers (3–→100), number of vascular bundles related to diameter of rhizome, brown-colored bundle sheaths present or absent. *Rhizome scales* clathrate, pseudo-peltate, narrowly deltoid, index 2–6, or round, diam. <0.1 mm (when dimorphic), with marginal protrusions and apical glandular trichome. *Phyllopodia* present, varying in size and distance, set with scales; scales similar to or slightly different from rhizome scales (as to indument and apex). *Roots* in two ventral rows, branched, dark brown, with unicellular, ferrugineous hairs. *Fronds* monomorphic, in two dorsal rows, articulate, petiolate, index 1.5-6.5. *Stripes* remote, diam. at base 1.5-5 mm, green, turning dorsally to dark-brown, basally set with scales, scales smaller than those of the rhizome; 4-20 vascular bundles, sclerenchyma sheaths present. *Lamina* (partly) pinnate, lateral segments articulate, terminal segments continuous; pinnae sessile or petiolate, lanceolate to linear, thin or firm herbaceous; venation goniophleboid, in apical part of pinnae (and in juvenile fronds) free; indument: scales and various types of hairs present. *Sori* in one row at each side of costa, terminal at included veins, superficial or impressed, round,diam. 0.8-2.1 mm; paraphyses glandular, hairy and scaly, peltate or palaceous. *Sporangia* with triseriate stalk, indurated annulus vertical, incomplete, stomium 2-celled, glandular sporangial paraphyses sometimes present, mostly absent; spores per sporangium 55-65, bilateral, oblong (polar view), plano concavo-convex (lateral view), light yellow; perispore smooth, cristately folded, exospore smooth or subverrucate.

Distribution Maps (Fig. 2)

1. *G. benguetense* (Copel.) Copel.
   G. persicifolium (Desv.) Bedd.
   G. pseudoconnatum (Copel.) Copel.
11. *G. mengtzeense* (Christ) Roedl-Linder
   G. percussum (Cav.) Wag. et Greth.
   G. terrestre Copel.
111. *G. subauriculatum* (Bl.) Presl

Key to the Species

1 a. Pinnae angustate at base 2
b. Pinnae truncate, cordate or auricled at base 4

2 a. Sori deeply impressed, surrounded by dark-brown paraphyses in ring-like arrangement; laminar trichomes acicular and glandular ..... 3. *G. percussum*
b. Sori superficial or slightly impressed, light-brown paraphyses scattered throughout the sori; laminar trichomes only glandular 3

3 a. Areolae in plural rows; exospore smooth ..... 4. *G. persicifolium*
b. Areolae in one row; exospore subverrucate. ..... 7. *G. terrestre*

4 a. Apical pinnae free; paraphyses palaceous ..... 1. *G. benquetense*
b. Apical pinnae adnate; paraphyses peltate 5

5 a. Rhizome slightly glaucous, rhizome scales dimorphic; paraphyses deltoid ..... 2. *G. mengtzeene*
b. Rhizome chalky white; rhizome scales monomorphic; paraphyses stellate. 6

6 a. Sclerenchyma strands in rhizome > 100; scales ferrugineous; sori diam. < 1.5 mm ..... 6. *G. subauriculatum*
b. Sclerenchyma strands in rhizome < 50; scales brunneous; sori diam. > 1.5 mm ..... 5. *G. pseudoconnatum*

Description of the Species

1. *Goniophlebium benguetense* (Copel.) Copel. (Fig. 3).

Goniophlebium benguetense (Copel.) Copel., F. Fl. Philipp. 3 (1960) 461. *Polypodium benguetense* Copel., Philipp. J. Sci. suppl. 1 (1906) 256; F. Fl. Philipp. 3 (1960) 461. *Schellolepis benguetensis* (Copel.) Pic. Ser., Webbia 28 (1973) 470. Type: E.B. Copeland 1829 ("1892"), Benguet, Ambuklao (MICH, holo).

Rhizomes 2.2-2.9 mm in diam., dark-green, sclerenchyma strands > 100, brown bundle sheaths absent. *Rhizome scales* spreading (appressed when old or dried), densely set, badious, index 2.5-4.5, 1.5-2 mm x 0.4-0.7 mm, marginal trichomes 1-cellular, hyaline (as in Fig. 13d). *Fronds:* index 2-4, 20-40(–50) x /-12(-18) cm. *Stipes:* length 1/2-1 of lamina, diam. at base 1.5-2.5 mm. *Laminae* widest at middle, rarely near base; lateral segments free, sessile or very shortly petiolate, (sub-) opposite, in basal part 1.2-2.5 cm apart, base truncate, apex pungent, margin coarsely serrate (see notes); terminal segment conform, rarely grown together with the first pair of lateral segments; rachis in apical part slightly winged; venation hardly visible (*in vivo*) costal areolae in 1 row; indument transient, trichomes acicular and glandular (Fig. 4.a & b); *Sori* 0.8-1.1 mm in diam., impressed; paraphyses persistent, scaly palceous or simply branched or filiform; sporangium 220-260 x 170-220 μm, indurated annulus cells 11 -13-(–14), epistomium cells 3, hypostomium cells 3, sporangial paraphyses absent spores 25.4-30.5 x 43.2-48.3 x 30.5-35.6 μm, perispore largely detached, exospore smooth.

Localities: Luzon, Provinces of Benguet, Bontoc, Ifugao, Ilocos Norte and Rizal. *Mindanao,* Lanao Prov. (Fresh material studied from all over the Mountain Provinces).

Habitat. Usually terrestrial, rarely epiphytic with the rhizome very tightly appressed; alt. 1000-1700 m.

Notes. 1. Plants growing in a sunny, dry place are smaller in size, with fronds that are lighter green, texture more firm, pinnae contracted, sori deeper impressed, margin less serrate; in the shade the margin can even be serrulate and the longest fronds pendent.

2. Copeland himself determined a few specimens of *G. benguetense* as *Goniophlebium integrum*, although *G. integrum* is exactly *G. subauriculatum*, which is very distinct from *G. benguetense*.

2. *Goniophlebium mengtzeense* (Christ) Roedl-Linder, comb. nov. (Fig. 5)

Goniophlebium mengtzeense (Christ) Roedl-Linder. *Polypodium (Goniophlebium) mengtzeense* Christ, Bull. Boiss. 6 (1898) 869; Hu et Ching, Ic. Fil. Sinic. 1 (1930) t. 42; Ching, Contr. Inst. Bot. Nat. Acad. Peiping 2, 3 (1933) 52; Copel., Philipp. J. Sci. 1 suppl. 2 (1906) 161, 256. Lectotype: A. Henry 10964 B, Yunnan, Mengtze (P).

Polypodium taiwanianum Hayata, Bot. Mag. 23 (1909) 80. *Marginaria taiwaniana* (Hayata) Nakai *ex* Itô, J. Jap. Bot. 11 (1935) 95. Type: T. Kawakami et U. Mori 2361, Taironkosha, n.v.

Rhizomes 3.5-4.7 (–5.5) mm in diam., subglaucous, frequently branched, sclerenchyma strands 40-80, brown bundle sheaths absent. *Rhizome scales* not densely set, spadiceous, dimorphic: I) spreading, ephemeral, 1.6-3.4 x 0.5-0.8 mm, II) appressed, persistent, at base of I, 0.06-0.1 mm in diam., marginal trichomes few, 2-cellular with brown gland (as in Fig. 11d). *Fronds:* index 2-4 (–6), 20-60 x 7-15 (–20) cm. *Stipes:* length (1/5–) 1/3– 4/5 of lamina, diam. at base (1.5–) 1.9- 2.7 mm. *Laminae* widest at middle; rachis ventrally flattened; apical lateral segments 1/3-2/3 of lamina adnate and partly decurrent, mostly opposite, in basal part 1.5-4.5 cm apart, free, sessile, base truncate, cordate or auricled; terminal segment 1/3 shorter or equally long as longest lateral segment, conform, apex pungent, margin subentire to crenate; costal areolae in 1 row; trichomes glandular (Fig. 4.a rarely b) persistent, acicular transient. *Sori* 0.8-1.5 mm in diam., superficial; paraphyses persistent, scaly peltate deltoid and few hairy; sporangium 250-270(–290) x 210-250 μm, indurated annulus cells (11–)13, epistomium cells 3, hypostomium cells 3, sporangial paraphyses absent; spores 30.5-34.3 x 48.3-50.8 x 27.9 38.1 μm, perispore most parts attached, exospore subverrucate.

Localities: Luzon, northern part. (Fresh material studied from Mt. Sto. Tomas and Mt. Pulog, Benguet Prov., and Mt. Data, Bontoc Prov.)

Habitat. Usually on mossy tree branches, rarely epilithic or terrestrial at rocky slopes; alt. 1900-2500 m.

Notes. 1. *G. mengtzeense* seems to be seasonal. During a field trip in November (end of rainy season) I found only yellow-brownish, all adult and fertile but over-mature fronds

with dark-yellow sori. In June (beginning of rainy season) all specimens found were fresh and light-green; many juvenile, also sterile fronds present, fertile fronds were not mature with pale-yellow sori. This plant hardly grows under lowland conditions.

2. The Mt. Pulog specimens appear to be slightly different: the margin of pinnae being more serrate, and the pinnae being situated alternately.

3. Philippine plants of this species have usually been identified as *G. argutum* (Wall. *ex* Hook.) J. Smith. They correspond with Christ's description of *Polypodium mengtzeense* from China. The study of the lectotype as well as of material from China, Taiwan and Mainland Asia indicate that these plants from the Luzon Highlands show conspicuous similarities to *P. mengtzeense* and *P. taiwanianum*. As far as I am concerned, the few, inconspicuous morphological differences do not jusitify a distinction on the species level.

3. *Goniophlebium percussum* (Cav.) Wag et Greth. (Fig. 6)

Goniophlebium percussum (Cav.) Wag et Greth., Occ. Pap. Bish. Mus. (2) 19 (1948) 88; Copel., F. Fl. Philipp. 3 (1960) 460; Price, Kalikasan Philipp. J. Biol. 1 (1972) 44. *Cyathea percussa* Cav., Descr. (1801) 548; C. Chr., Ark. f. Bot. (11) 9 (1910) 38, Dansk. Bot. Ark. (3) 9 (1937) 29. *Polypodium percussum* (Cav.) Kaulf., Enum. Fil. (1924) 90. *Schellolepis percussa* (Cav.) Pic. Ser., Webbia 28 (1973) 470. Type: not seen.

Polypodium cyathoides Sw., Syn. Fil. (1806) 37; C. Chr., Ind. Fil. (1906) 520; Ark. f. Bot. (11) 9 (1910) 38, 39; Dansk. Bot. Ark. (3) 9 (1937) 29. *Goniophlebium cyathoides* (Sw.) Hosok, Trans. Nat. Hist. Soc. Formosa 32 (1942) 286; C. Chr., Ind. Fil. suppl. (1960).

Polypodium verrucosum (Wall., Cat. Nr. 296 (1828) nom. nud.); Hook., Gard. F. (1862) t. 41; Sp. Fil. 5 (1864) 31; Syn. Fil. (1883) 344; Diels, NPf (1902) 312; C. Chr., Ind. Fil. (1906) 573; Dansk. Bot. Ark. (3) 9 (1937) 29; Copel., Philipp. J. Sci. (1) 2 (1907) 139; Holtt., Fl. Mal. 2 (1955) 206 & fig. 106. *Marginaria verrucosa* (Wall *ex* Hook.) Prsl., Tent. (1836) 186; Hook., Gen. Fil. (1838) t. 14; Fee, Con. (1850-52) 255; J. Smith, F. Brit. & For. 1 (1866) 83. *Goniophlebium verrucosum* (Wall. *ex* Hook.) J. Smith, Cat. (1857) 4; F. Brit. & For. (1866) 83; apud Hook., Gen. Fil. (1840) note to t. 51; Bedd., Handb. (1833) 324; Handb. suppl. (1892) 91; Nayar, Comp. Bedd. Handb. (1974) 79. *Schellolepis verrucosa* (Wall. *ex* Hook.) J. Smith apud Hook., Gen. Fil. (1840) note to t. 51; F. Brit. & For. (1866) 83; Hist. Fil. (1875) 93; C. Chr., (1906) 616.

Polypodium cuspidatum Mett., Pol. (1857) 81, non Don (1825) nec Prsl. (1825), nec Bl. (1828); Bot. Zeit. 4 (1846) 423; Linn. 23 (1850) 277; Hook., Sp. Fil. 5 (1864) 32; C. Chr., Ind. Fil. (1906) 520.

Rhizomes 5-11 mm in diam., light-green, sclerenchyma strands 70-100; brown bundle sheaths present. *Rhizome scales* appressed, densely set or ephemeral, index 2.8-3.6 (–6.5), 2.5–4 x 0.7–1.3 mm, rhizoid hairs attached to surface, marginal trichomes absent. *Fronds:* index 1.5-3.5 (–4.5), 38–110(–200) x 18–38(–45) cm. *Stipes* can turn

dark-brown also ventrally in old fronds, length 1/5–1/2(–3/5) of lamina, diam. at base 3.3–4.9 mm. *Laminae* widest near base; all segments conform, shortly petiolate, base short angustate, apex acuminate to rostrate, margin sub-entire to crenate; lateral segments (sub–) opposite, in basal part 2–4(–6) cm apart; costal areolae in 2–3 rows; trichomes acicular and glandular (Fig. 4d) persistent. *Sori* 0.8–1.0 mm in diam., deeply impressed (Fig. 7a); paraphyses persistent, dimorphic: 1) simple, narrow, index 3–4, palaceous, 11) branched or scale-like, index 1–2, peltate, dark-brown, ringlike arranged around each sorus; sporangium 210–300 x 170–205 μm, indurated annulus cells (12–) 13–14, epistomium cells 3, hypostomium cells 3, sporangial paraphyses absent; spores 20.3–25.4 x 38.1–44.5 x (20.3–)25.4–34.3 μm, perispore most parts attached, exospore smooth.

Localities: Luzon, southern part, *Samar*, *Mindanao*. (Fresh specimens studied from U.P. Land Grant, Quezon Prov.).

Habitat. In rain forests, epiphytic on rotten tree trunks and terrestrial in loose soil; alt. 100–1200 m.

Notes. The fertile pinnae are sometimes contracted and drooping, the rachis being very flexible. This species is otherwise in aspect very similar to *G. persicifolium*, the sterile fronds even being hardly distinguishable; the distinguishable characters for these two species are presented in Table 1.

Table 1. Diagnostic characters to distinguish *G. percussum* and *G. persicifolium*

Characters	*G. percussum*	*G. persicifolium*
Sclerenchyma strands in rhizome	70–100	40–50
Rhizome scales	Marginal trichomes absent	Marginal trichomes present
Laminar indument in adult plants	Trichomes acicular & glandular	Trichomes only glandular
Sori	Diam. <1.5 Completely impressed Ring of dark paraphyses present	Diam. >1.5 Globose Ring of dark paraphyses absent

4. *Goniophlebium persicifolium* (Desv.) Bedd. (Fig. 8)

Goniophlebium persicifolium (Desv.) Bedd., F. Brit. Ind. Corr. (1870) (?); Copel., F. Fl. Philipp. 3 (1969) 459. *Polypodium persicifolium* Desv., Berl. Mag. 5 (1811) 316; Hook., Syn. Fil. (1883) 344; Holtt., Fl. Mal. 2nd ed. (1954) 206; C. Chr., Ind. Fil. (1906) 552. Copel., F. Fl. Philipp. (1960) 459. *Schellolepis persicifolia* (Desv.) Pic. Ser., Webbia 28 (1973) 470. Type: not seen.

Polypodium cuspidatum Bl. nec Don, 1825, nec Prsl., 1825, Enum. Plant. Jav. 1 (1827) 132, 133; Fil. Jav. Pl. 82 (1828) 175; Hook., Sp. Fil 5 (1864) 32; C. Chr. Ind. Fil. (1906) 520. *Goniophlebium cuspidatum* (Bl.) Prsl., Tent. (1836) 186. *Schellolepis cuspidata* (Bl.) J. Smith, F. Brit. & For. (1866) 82; Hist. Fil. (1875) 93; C. Chr., Ind. Fil. (1906) 616.

Polypodium grandidens Kze., Bot. Zeit. (1846) 423; Ind. Fil. H. Bot. Lips. (1843) 33 t. 23; Linn. 23 (1850) 318, 319; Fée, Gen. (1850-52) 255; Hook., Sp. Fil. 5(1850) 31; J. Smith, F. Brit. & For. (1866) 82. C. Chr., Ind. Fil. (1906) 531. *Goniophlebium grandidens* (Kze.) Fee, Gen. (1850-52) 255.

Polypodium colpothrix Kze., Linn. 23 (1850) 276, 316; C. Chr., Ind. Fil. (1906) 517.

Polypodium phlebodioides Copel., Polyp. Philipp. (1905) 123; Philipp. J. Sci. 1, suppl. 2 (1906) 162; F. Fl. Philipp. 3 (1960) 459; C. Chr., Ind. Fil. (1906) 553. Type: E.B. Copeland 1762 a, Mindanao, Mt. Apo (MICH, holo).

Polypodium integrius ("integriore") Copel., Philipp. J. Sci. (1) 2 (1907) 139; F. Fl. Philipp. 3 (1960) 459. Type: E.D. Merrill 6005, Mindoro, Mt. Halcon (PNH, holo destroyed in 1945; MICH, iso).

Polypodium koningsbergeri v.A.v.R., Bull. Dépt. Agric. Ind. néerl. 18 (1908) 21 C. Chr., Ark. f. Bot. (11) 9 (1910) 38 anks. Bot. Ark. (3) 9 (1937) 29.

Rhizome 5-10 mm in diam., light-green, sclerenchyma strands 40-50, brown bundle sheaths present. *Rhizome scales* appressed, densely set or ephemeral, ferrugineous, index 1.9-3.4, 3-4 x 0.9–1-5 mm, unicellular, light-brown hairs attached to surface, marginal trichomes many, 1-cellular, hyaline. *Fronds:* index 2.5-3.5(–4.0), (35–)60–150 x (14–)20–38 cm. *Stipes:* length 2/5–3/4 of lamina, diam. at base 2.8-4.5 mm. *Laminae* widest at base or equally wide all along; all segments conform, petiolate, in basal part 3-4.5(–6) cm apart, base angustate, apex rostrate, margin (sub–) entire to crenate; costal areolae in 2-3 rows (Fig. 9); trichomes glandular (Fig. 4. a, b, c) persistent, acicular transient. *Sori* 1.8–2.1 mm in diam., globose, superficial or slightly impressed (Fig. 7b); paraphyses transient, light-brown, simply branched or scale-like, peltate, hairy glandular; sporangium 235-276 x 153-194 μm, indurated annulus cells 12-13, epistomium cells 3, hypostomium cells 3, sporangial paraphyses absent; spores 20.3–24.2 x (34.3–) 38–43.2 x 22.8–28 μm, perispore largely attached, exospore smooth.

Localities: Luzon, southern part; *Mindanao.* (Fresh material studied from Mt. Makiling, Laguna Prov. and U.P. Land Grant, Quezon Prov.).

Habitat. In rain forests, usually epiphytic on rotten or living tree trunks, rarely terrestrial in loose soil; alt. 300-1200m.

Note. Some mature plants do not develop long, pendent fronds but only short, upright ones.

5. *Goniophlebium pseudoconnatum* (Copel.) Copel. (Fig. 10)

Goniophlebium pseudoconnatum (Copel.) Copel., F. Fl. Philipp. 3 (1960) 462. *Polypodium pseudoconnatum* Copel, non Wu, 1932. Philipp. J. Sci. suppl. (1906) 161 t. 22; F.Fl. Philipp. 3 (1960) 462; Price, MS Thesis U.P. Los Baños (1975) 204. Lectotype; E.B. Copeland 1904 a, Lepanto, Bagnen (MICH).

Rhizomes 5-7.5 (-10.5) mm in diam., chalky white, sclerenchyma strands 30(50) (Fig. 11a), brown bundle sheaths present. *Rhizome scales* spreading, ephemeral, brunneous, index 3.2-4.1, 4-5 x 1.0–1.3 mm, thickly clathrate, cells white translucent, marginal protrusions equally long at base and apex, marginal trichomes many, 2-cellular, glandular (Fig. 11d), same type of trichomes can be present at basal part of surface. *Fronds:* index 2.5–4(–5.5), 26–150 x 8–31 cm, dark-green. *Stipes:* length 2/5–3/4(–3/5) of lamina, diam. at base 2.5–4.3 mm. *Laminae* index 1.9–2.5, widest near base or equally all along; lateral segments in apical part adnate, others free, sessile, (sub–) opposite or alternate, in basal part 1.5–3.5 cm apart, base truncate, cordate or auricled, apex pungent, margin crenate; terminal segment difforme; marginal serration getting deeper from apex to base and turning into lateral segments; costal areolae in one row; trichomes acicular and glandular (Fig. 4b) persistent. *Sori* 1.5–2 mm in diam., superficial but distinguishable on upper surface; paraphyses persistent, scaly stellate peltate and hairy; sporangium 214–235 x 153–164 μm, indurated annulus (11)12 cells, epistomium (2) 3 cells, hypostomium 3 cells, sporangial paraphyses sometimes present, 2(–3)–cellular, glandular, attached to 1st (2nd) epistomium cell; spores 22.9–26.7 x 39.4–47.0 x 21.5–30.5 μm, perispore largely attached, exospore subverrucate.

Localities: Luzon; Provinces of Ifugao, Bontoc, Benguet and Quezon. (Fresh material studied from Northern part of Luzon).

Habitat. Epiphytic on mossy tree trunks, epilithic on mossy rocks, sometimes rhizome is growing away from any substrate up to 30 cm free in the air alt. 1200-2100 m.

Notes. 1. The frond outline of *G. subauriculatum* and *G. pseudoconnatum* is similar; the medial to lower pinnae of *G. pseudoconnatum* are mostly "more strictly opposite and more cordate, thus appearing connate" (Copeland, 1960 p. 462). The following differences (Table 11) justify a distinction into two species:

Table 2. Diagnostic characters to distinguish *G. pseudoconnatum* and *G. subauriculatum*

Character	*G. pseudoconnatum*	*G. subauriculatum*
Sclerenchyma strands in rhizome	30(50)	100
Rhizome scales	Brunneous	Ferrugineous
Marginal trichomes	2-cellular incl. 1 brown gland	1-cellular hyaline
Stipe and rachis	Slightly flexible therefore fronds upright or pendent	Very flexible therefore fronds drooping
Lamina	Dark-green	Light-green
Sori	Diam. 1.5–2 mm	Diam. 1–1.5 mm
Sporangium	Glandular paraphyses can be present	Paraphyses absent

2. I have collected both species side by side in the mossy forest of Mt. Sto. Tomas at 2000 m. *G. pseudoconnatum* hardly grows under lowland conditions, whereas the "mountain form" of *G. subauriculatum* (see notes to species 6) develops very well in my Manila garden at 30 m alt.

6. Goniophlebium subauriculatum (Bl.) Prsl (Fig. 12)

Goniophlebium subauriculatum (Bl.) Prsl, Tent. (1836) 186; J. Smith, J. Bot 3 (1841) 396; Bedd., F. Brit. Ind. (1866) pl. 78; Copel., F. Fl. Philipp. 3 (1960) 461; Nayar, Comp. Bedd. Handb. (1974) 79. *Polypodium subauriculatum* Bl., Enum. Plant. Jav. 1 (1877) 133; Fil. Jav. (1828) t. 83; Mett., Pol. (1857) 81; Hook., Sp. Fil. 5 (1864) 32; Syn. Fil. (1883) 344; Clarke, Trans. Linn. Soc. Bot. 1 (1880) 551; Bedd., Handb. (1883) 322, 323 t. 173; Handb. (1892) 90. Christ, Bull. Boiss. 6 (1898) 198; C. Chr., Ind. Fil. (1906) 567; Ching, Contr. Inst. Bot. Nat. Acad. Peiping 2,3 (1933) 51. Holtt., Fl. Mal. 2 (1955) 207 fig. 108; Copel., F. Fl. Philipp. 3 (1960) 461; Philipp. J. Sci. (1) 2 (1907) 139. var. *serratifolium* Hook., Sp. Fil. 5 (1864) 33; Copel., F.Fl. Philipp. 3 (1960) 462. *Schellolepis subauriculata* (Bl.) J. Smith, F. Brit. & For. (1866) 82; Hist. Fil. (1875) 93; C. Chr., Ind. Fil. (1906) 616. Type: Blume (?) s.n., s.d. Java (130), Bantam (129) (L, holo and iso).

Polypodium pallens Bl., Fil. Jav. (1828) 178 t. 84 fig. 1; Mett., Pol. (1857) 81; Hook., Sp. Fil. 5 (1864) 33; Copel., Philipp. J. Sci. suppl. 2 (1906) 162; C. Chr., Ind. Fil. (1906) 550. *Goniophlebium pallens* (Bl.) Prsl, Tent. (1836) 186 (G?); J. Smith, J. Bot. 3 (1841) 396; Feé, Gen. (1850-52) 255; Copel., F. Fl. Philipp. (1960) 461. *Schellolepis pallens* (Bl.) J. Smith, Hist. Fil. (1875) 93; C. Chr., Ind. Fil. (1906) 616. Type: Kuhl et van Hasselt 206 (L, holo).

Goniophlebium reinwardtii de Vriese, Ned. Kr. Arch. 1 (1947) 257; Feé, Gen. (1850-52) 255. C. Chr., Ind. Fil. (1905) 327. *Polypodium reinwardtii* (de Vriese) Kze., Linn. 23 (1850) 283; J. Smith, F. Brit. & For. (1866); C. Chr., Ind. Fil. (1906) 559.

Goniophlebium pleopeltis Fee, Gen. (1850-52), 255, 256; J. Smith, F. Brit. & For. (1866) 82. Type: Lobb 263, Java, n.v.

Polypodium beddomei Bak. in Hook., Syn. ed. 2 (1883) 344; C. Chr., Ind. Fil. (1906) 513; Copel., Philipp. J. Sci. 1 suppl. 2 (1906) 162.

Goniophlebium molle Bedd., F. Brit. Ind. 2 (1868) 206 t.; Handb. (1883) 322 & t. 172; Handb., (1892) 90; Ching, Contr. Inst. Bot. Nat. Acad. Peiping 2, 3 (1933) 50, 51; Copel., F. Fl. Philipp. 3 (1960) 462; Nayar, Comp. Bedd. Handb. (1974) 79. Type: Burma n.v.

Polypodium mollicuium Copel., (non Kze. 1841), Perkin's Fragm. 3 (1905) 190; Philipp. J. Sci. suppl. 2 (1906) 162; F. Fl. Philipp. 3(1960) 461; C. Chr., Ind. Fil. (1906) 545 Type: Elmer 6506, Baguio (PNH, holo destroyed in 1945).

Polypodium beddomei Copel., Philipp. J. Sci. 1 suppl. 2 (1906) 162, *nom. nud.*; F. Fl. Philipp. 3 (1960) 462.

Goniophlebium integrum Copel., Philipp. J. Sci. (1) 81 (1952) 42; F. Fl. Philipp. 3 (1960) 461. Price, Kalikasan, Philipp. J. Biol. 1 (1972) 44. (Type: E.D. Merrill 7814, Luzon, Benguet, (MICH, holo).

Polypodium tomentellum (C. Chr., Ind. Fil. (1906) 570, nom. nud.) Copel., F. Fl. Philipp. 3 (1960) 461. *Goniophlebium tomentellum* (C. Chr. *ex* Copel.) Copel., F. Fl. Philipp. 3 (1960) 461, 462. *Schellolepis tomentella* (C. Chr. ex Copel.) Pic. Ser., Webbia 28 (1973) 470; Price, MS Thesis (1975) 204; C. Joaquin, PhD Thesis U.P. Diliman (1986) 143.

Rhizomes 5–6.5(–10) mm in diam., chalky white, sclerenchyma strands> 100 (Fig. 13a), brown bundle sheaths present. *Rhizome scales* spreading when fresh, densely set or ephemeral, ferrugineous, index 3.5–6, 3–5 x 0.6–1 mm, thinly clathrate, cells white translucent, single yellow cells in-between, often rhizoid hairs at surface; marginal protrusions longer at base than at apex, marginal trichomes few, 1-cellular, hyaline (Fig. 13d). *Fronds:* index 2.5–5(–6.5), 20–170(–300) x 7–45(–50) cm, light-green. *Stipes* can turn dark-brown also ventrally in adult fronds, length 1/5–3/4 of lamina, diam. at

base 1.8–2.2 mm. *Lamina:* index (1.2–) 1.8 (–8.5), widest at middle or at base or equally wide all along; lateral segments at apical part adnate, others free, sessile or shortly petiolate, (sub–) opposite or alternate, at basal part 1.5–3.5 cm apart, base truncate, cordate or auricled, apex pungent, margin crenate to serrate; terminal segment difforme (like *pseudoconnatum*); costal areolae in (1–)2(–3) rows; trichomes acicular and glandular (Fig. 4b) transient or persistent. *Sori* 1–1.5 mm in diam., impressed; paraphyses persistent or transient, scaly peltate stellate, hairy, sporangium 193-225 x 163-189 μm, indurated annulus 12(13) cells, epistomium 3 cells, hypostomium 3(4) cells, paraphyses absent; spores 20.3-25.4 x 35.5-39.4 x 22.8-25.4 μm, perispore largely attached, exospore subverrucate.

Localities: all over *Luzon, Negros, Mindoro, Mindanao.* (Fresh material studied from all over Luzon and the whole altitudinal range).

Habitat. Epiphytic on rotten and living tree trunks, epilithic or in clefts between rocks; alt. 250-2000 m.

Notes. 1. Having seen the type specimen of *G. integrum* and relevant herbarium and fresh material from the type locality of *G. tomentellum*, I am convinced that these two species are mere forms of the strikingly variable *G. subauriculatum*. Copeland's main distinctive character for *G. tomentellum* being persistently hirsute could not be confirmed. Juvenile plants of *G. subauriculatum* are hairy and the hairiness may persist to some extent in older plants. I have seen every gradation in hairiness even in adult fronds growing at the same rhizome.

2. Above 1500 m *G. subauriculatum* appears as a kind of "mountain form" which seems to be *P. pallens* Bl. It does not develop pendent or drooping fronds and the pinnae are narrow with a firm texture, a truncate base and a less serrate margin. Growing these specimens at an altitude of 30 m, the newly developed fronds look like any *G. subauriculatum* specimen collected from the lowlands: long, drooping fronds with very flexible rachis, pinnae thin-herbaceous with the margin serrate sometimes even serrulate and more auricled base.

7. *Goniophlebium terrestre* (Copel.) Copel. (Fig. 14)

Goniophlebium terrestre (Copel.) Copel, Philipp. J. Sci. (1935) 106 t. 13, 14; F. Fl. Philipp. 3 (1960) 460; Price, Philipp. Agric. 57 (1974) 44. *Schellolepis terrestris* Copel Price, Kalikasan Philipp. J. Biol. 3 (1974) 178; Joaquin, PhD Thesis U.P. Diliman (1986) 142. Type: E.B. Copeland (PPE) 272, Mt. Makiling (PNH, holo destroyed in 1945; MICH, iso).

Rhizomes dorsiventrally flattened, 1.8–3 x 1.5–2.6 mm in diam., dark-green, rarely branching, sclerenchyma strands 3–11, brown bundle sheaths present. *Rhizome scales* appressed when fresh, spreading when exsiccated, densely set, index 3.5-5, 3-4.7 x 0.8–1.1 mm, marginal protrusions short at apex, longer and lacerate at base, less clathrate towards base, marginal trichomes few, 1–2–cellular, glandular. *Fronds:* index 1.3–3 15–25 (38) x 8 –14 cm. *Stipes:* length 1/2–3/4 (–4/5) of lamina, diam. at base 1.1–1.8

mm. *Laminae* widest at base; all segments conform, shortly petiolate, (sub–)opposite, at basal part 1.3–2.5 mm apart, base angustate, apex rostrate, margin subentire to serrate; rachis slightly winged; costal areolae in 1 row, marginal veins very short, all free. trichomes glandular (Fig. 4b) persistent, acicular absent. *Sori* 1.6–2 mm in diam., superficial; paraphyses persistent, palaceous branched, peltate scaly; sporangium 285–342 x 204–245 μm, indurated annulus cells 13(14), epistomium cells 3, hypostomium cells 3, paraphyses absent; spores 27.9–38.1 x 45.7–58.4 x 33–43.2 μm, perispore largely attached, folds building winglike appendages at poles, exospore subverrucate.

Locality: ENDEMIC, confined to Mt. Makiling, Laguna Prov., *Southern Luzon.*

Habitat. Lower portion of mossy forest, terrestrial but as frequently epiphytic on tree trunks or epilithic on mossy or blank rocks; alt. 500-850.

Notes. 1. In young fronds not only the rachis is winged but also the stipe.
2. The present species is small-sized with a thin rhizome. Planted in my Manila garden more than a year ago, it developed very well but has not produced any fertile fronds as yet.

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Fig. 1. Spore of G. subauriculatum *(X 2,000)*

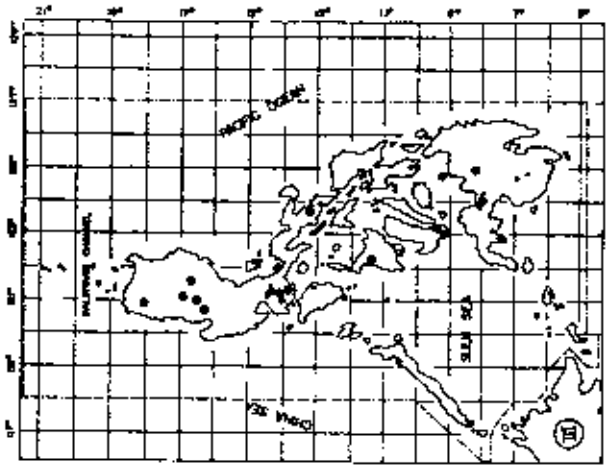

Fig. 2 – III

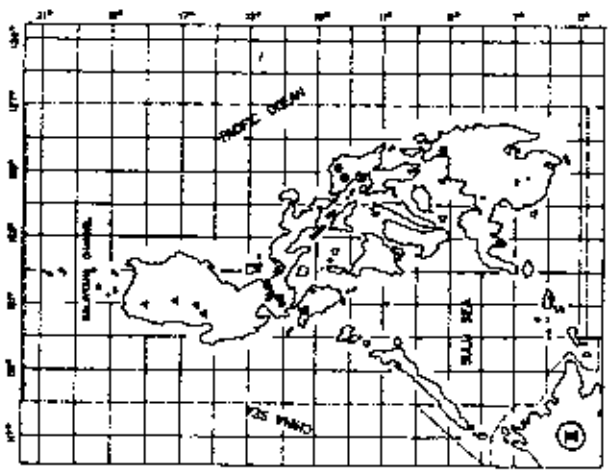

Fig. 2 – II

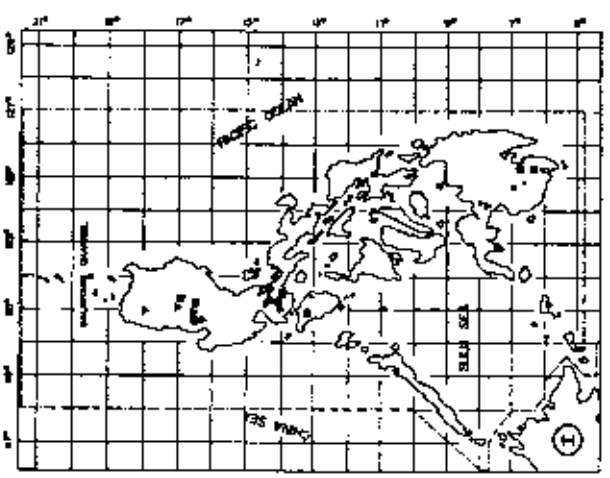

Fig. 2 – I

Fig. 2. Distribution Maps

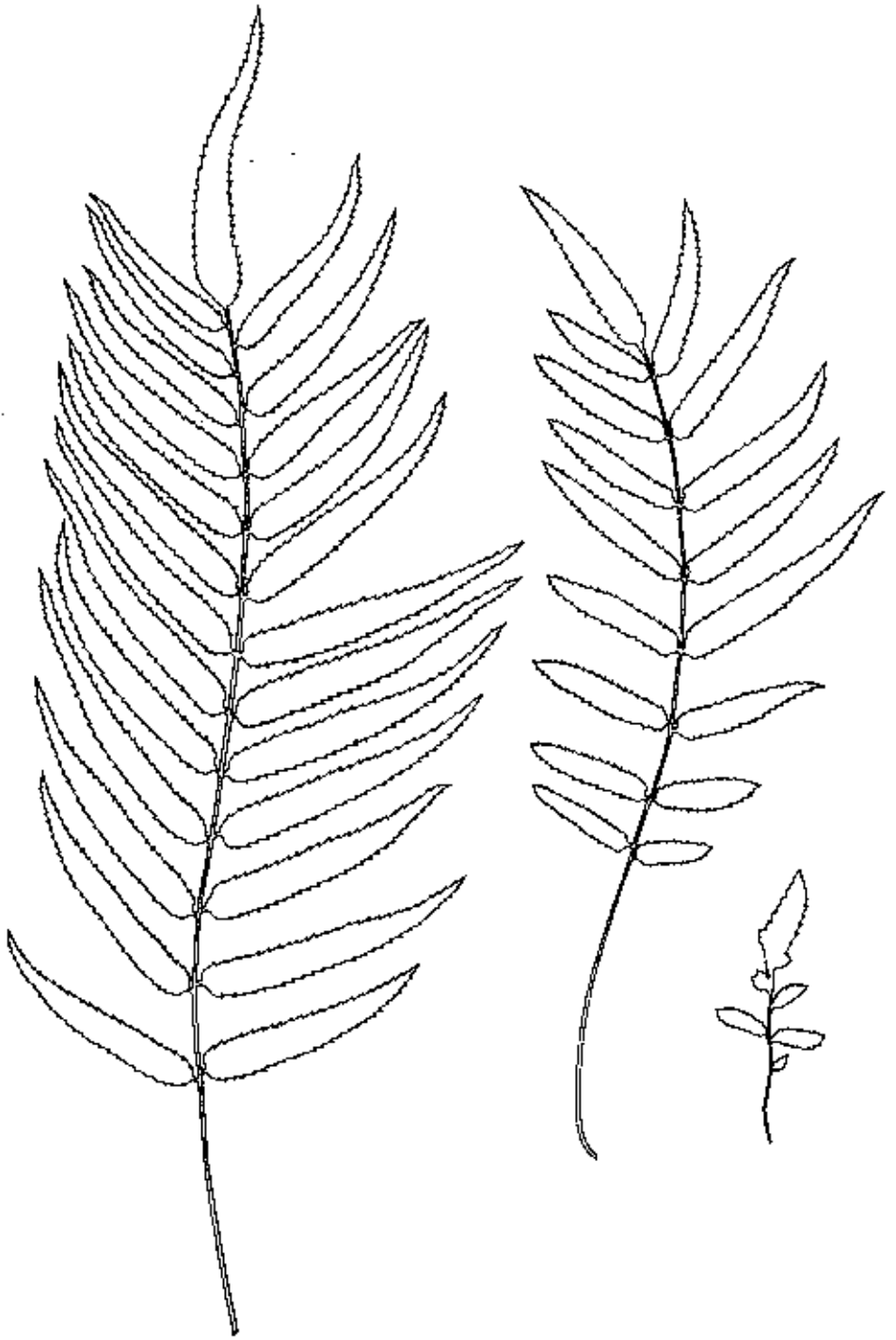

Fig. 3. Outline of G. benguetense[+] *(Copel.) Copel.*
+ Heteroblastic leaf series

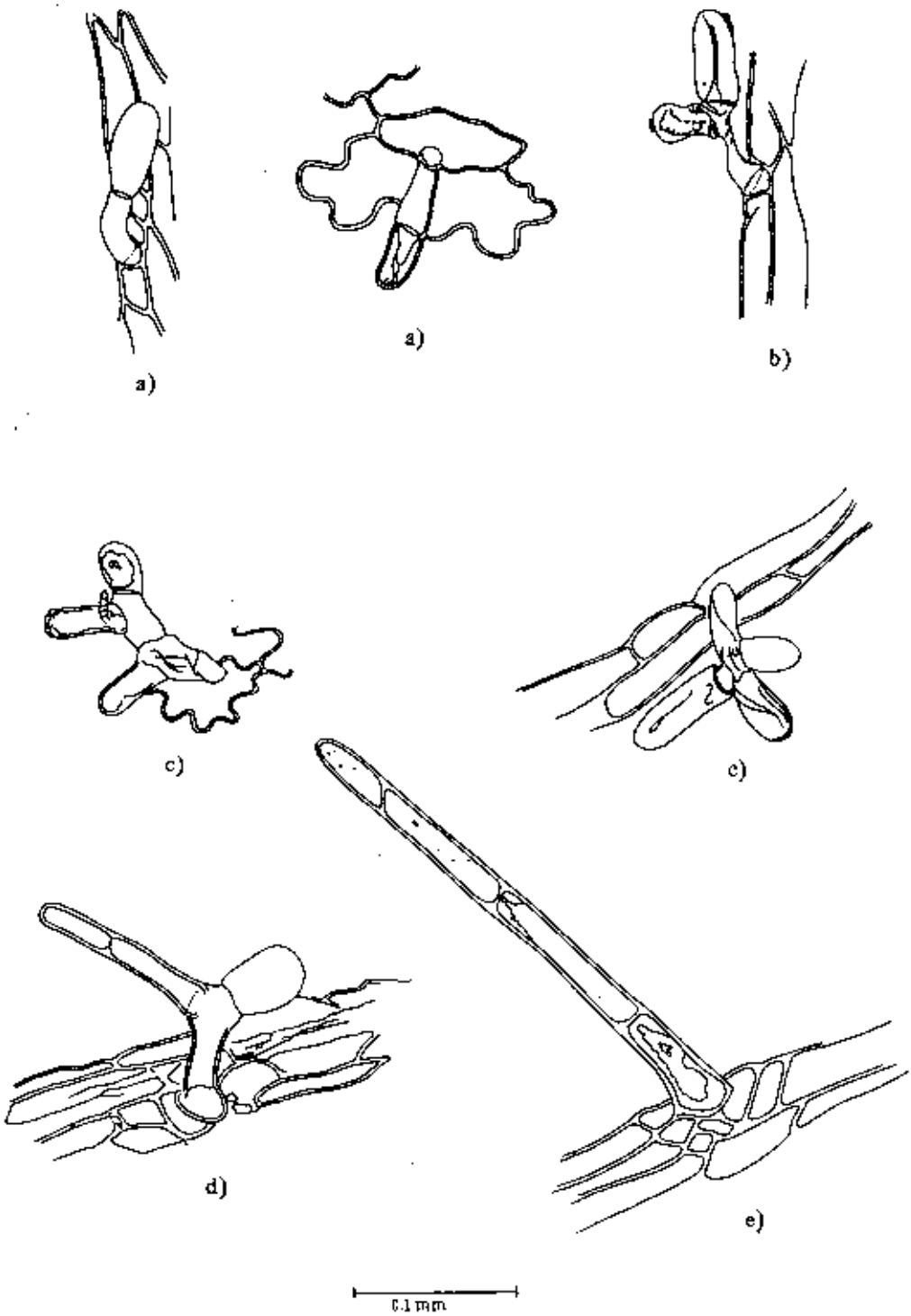

Fig. 4. Frond indument, a), b). c), glandular d) glandular and acicular, e) acicular.

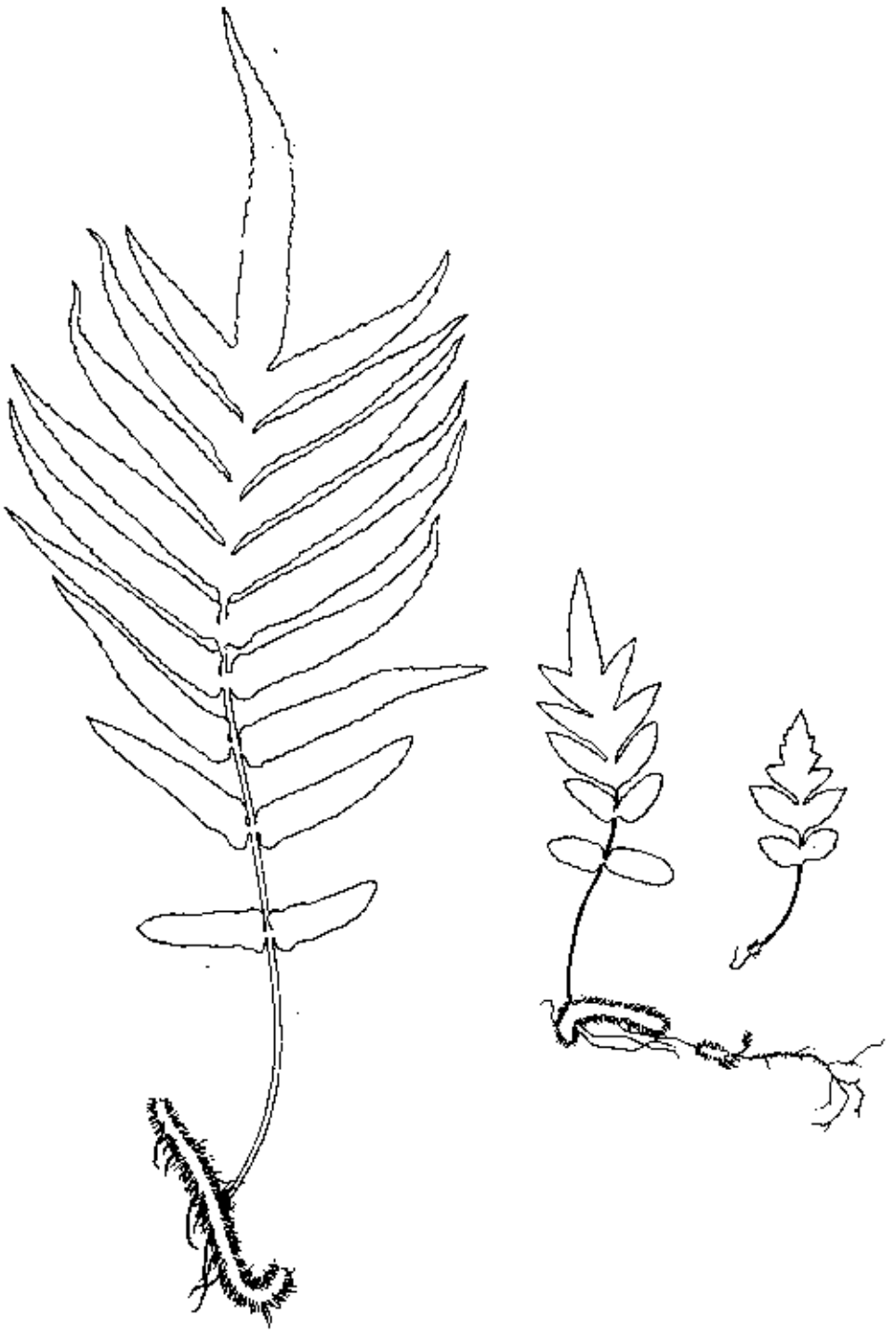

Fig. 5. Outline of G. mengtzense[+] *(Christ) Roedl – Linder*
+ Heteroblastic leaf series

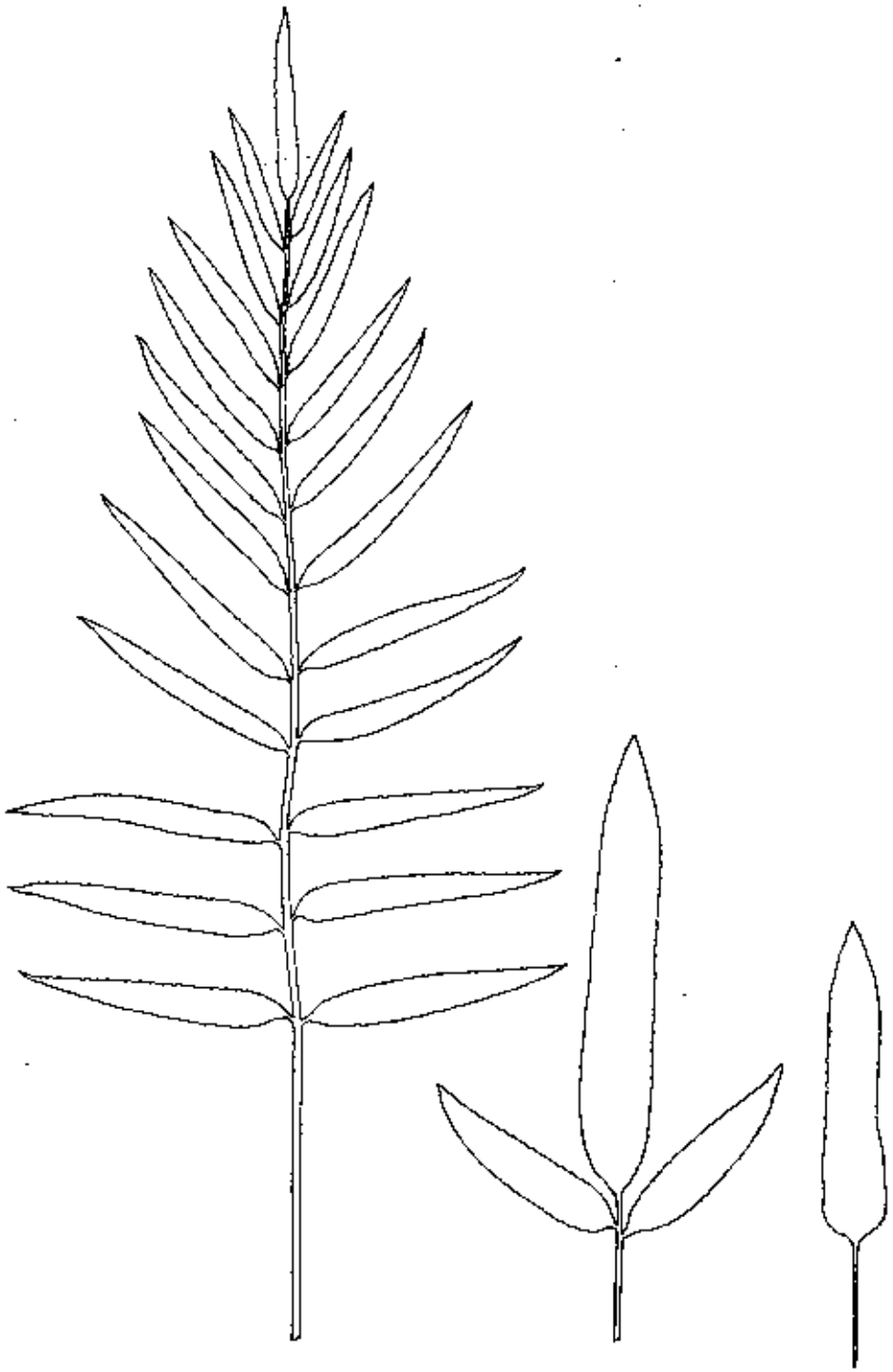

Fig. 6. Outline of G. percussum[+] *(Cav.) Wag. et. Greeth*

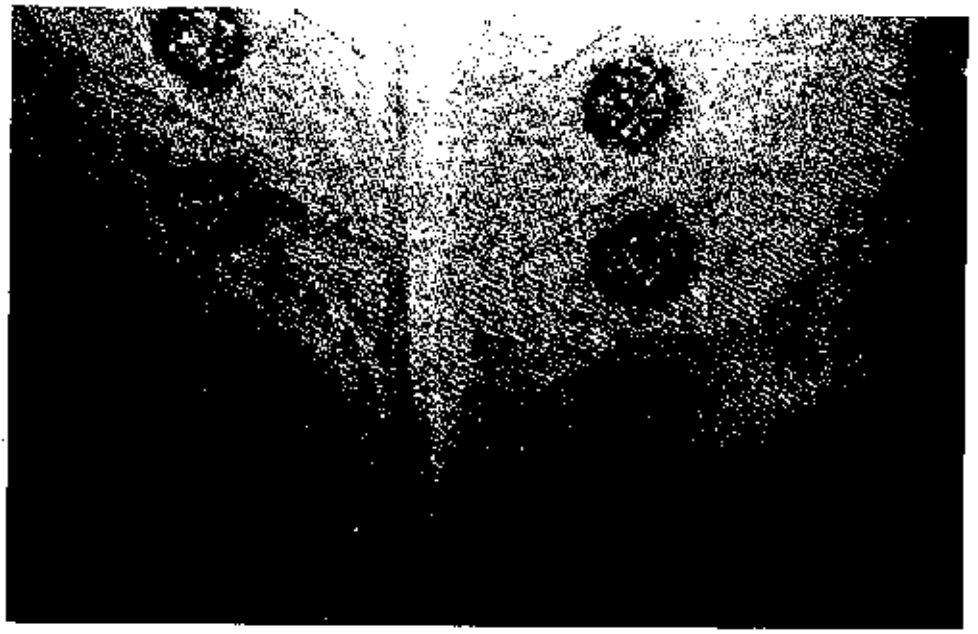

Fig. 7a. Sori of G. percussum *(X 6.3)*

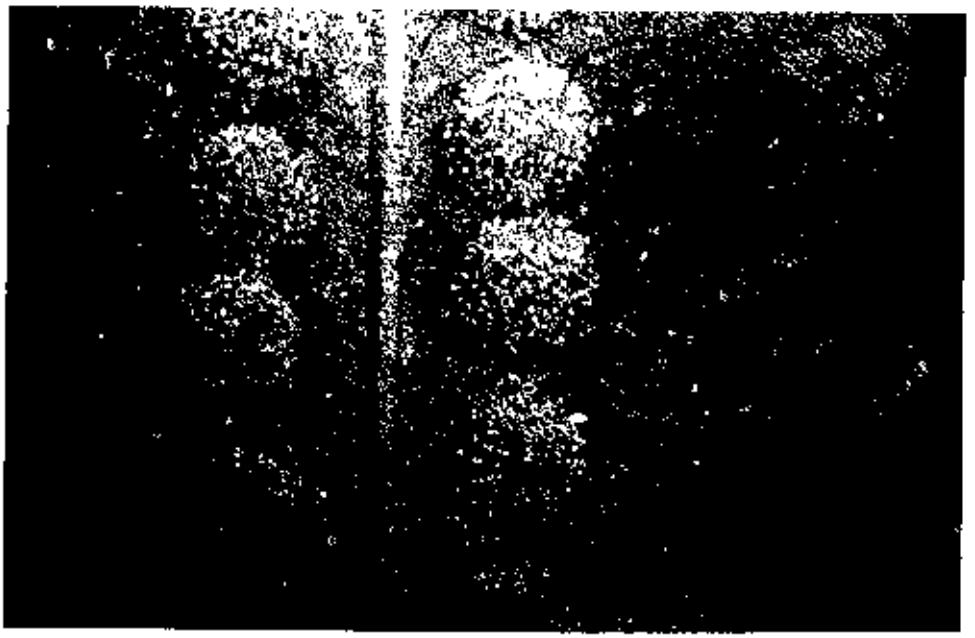

Fig. 7b. Sori of G. persicifolium *(X 6.3)*

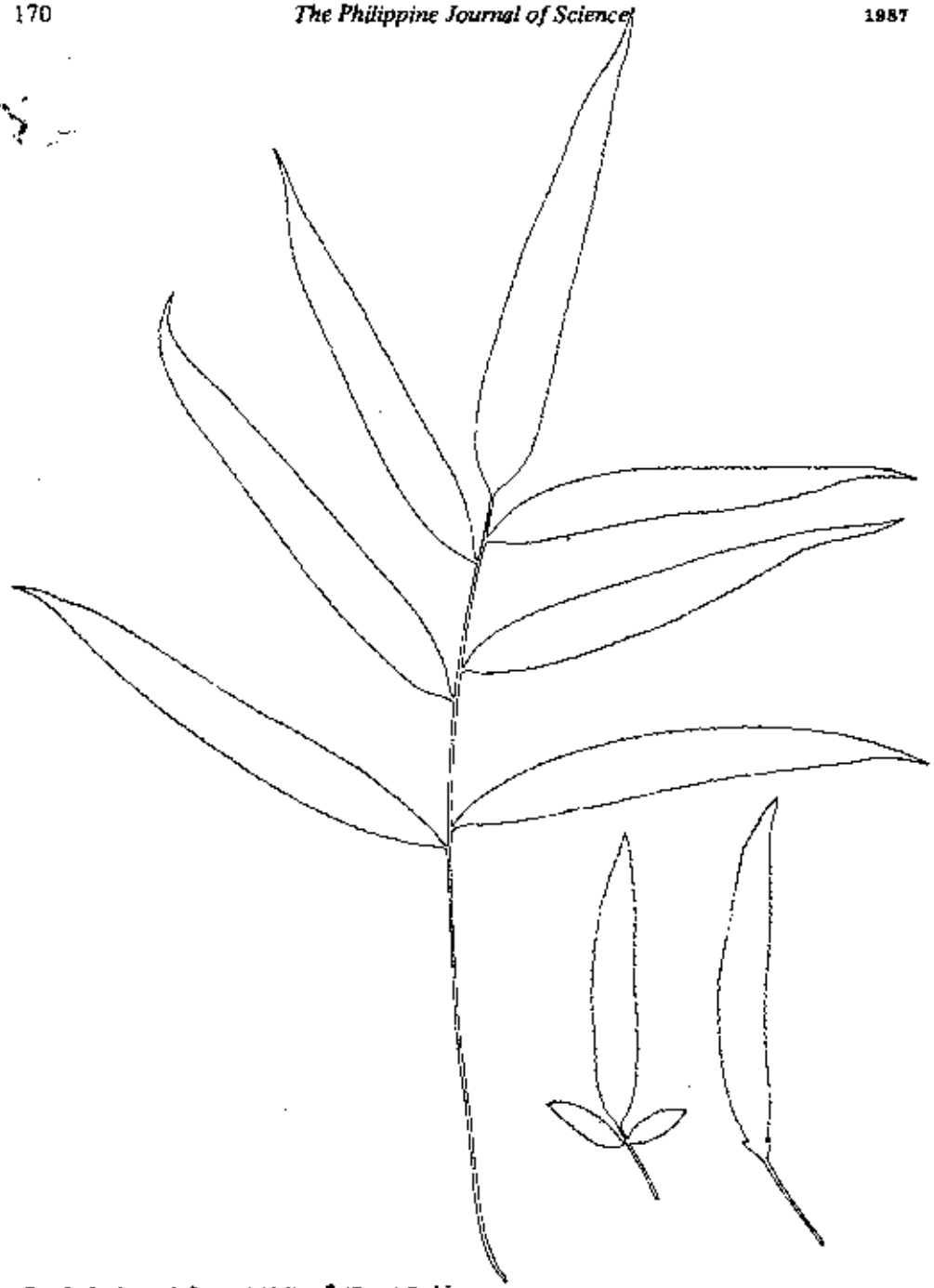

Fig. 8. Outline of G. persicifolium* *(Desv) Bedd.*
+ Heteroblastic leaf series

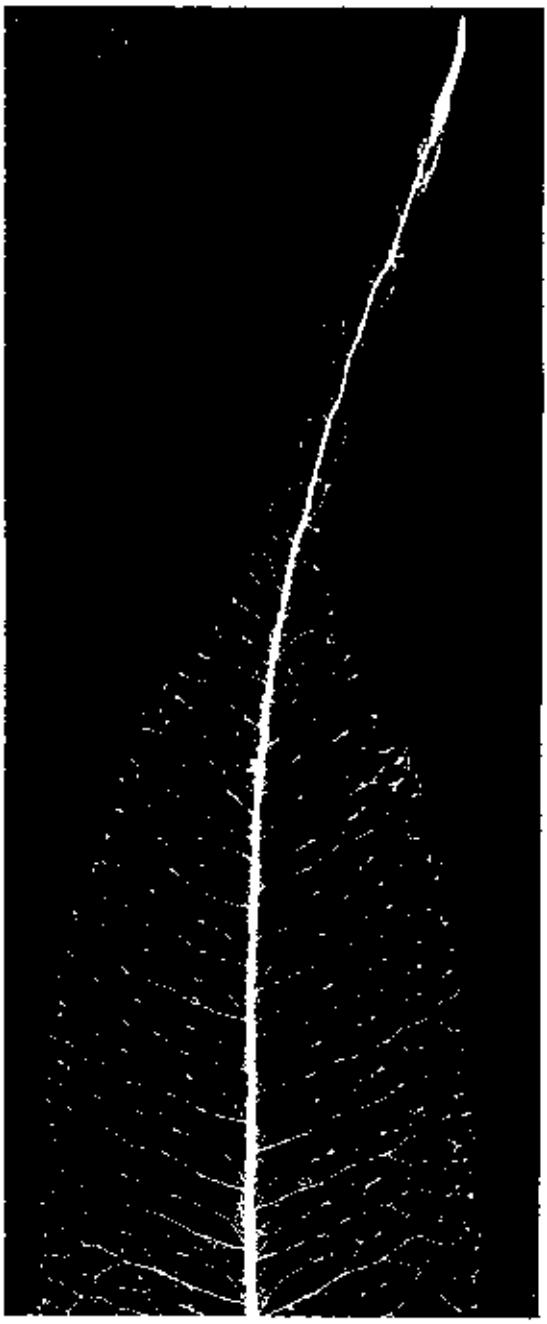

Fig. 9. Venation of G. persicifolium *(X 2.75)*

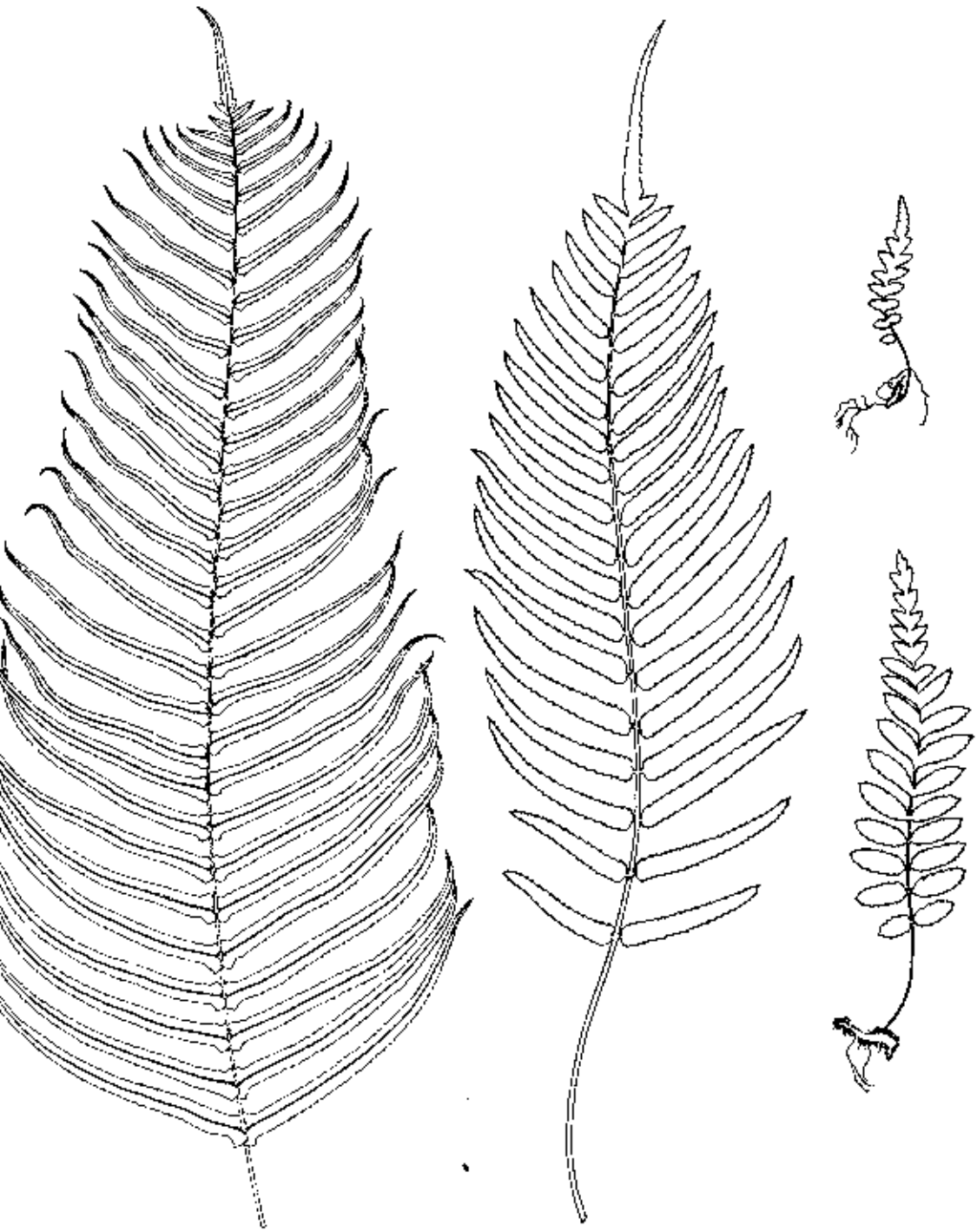

Fig. 10 Outline of G. pseudoconnatum* *(X 0.25)*
** Heteroblastic leaf series*

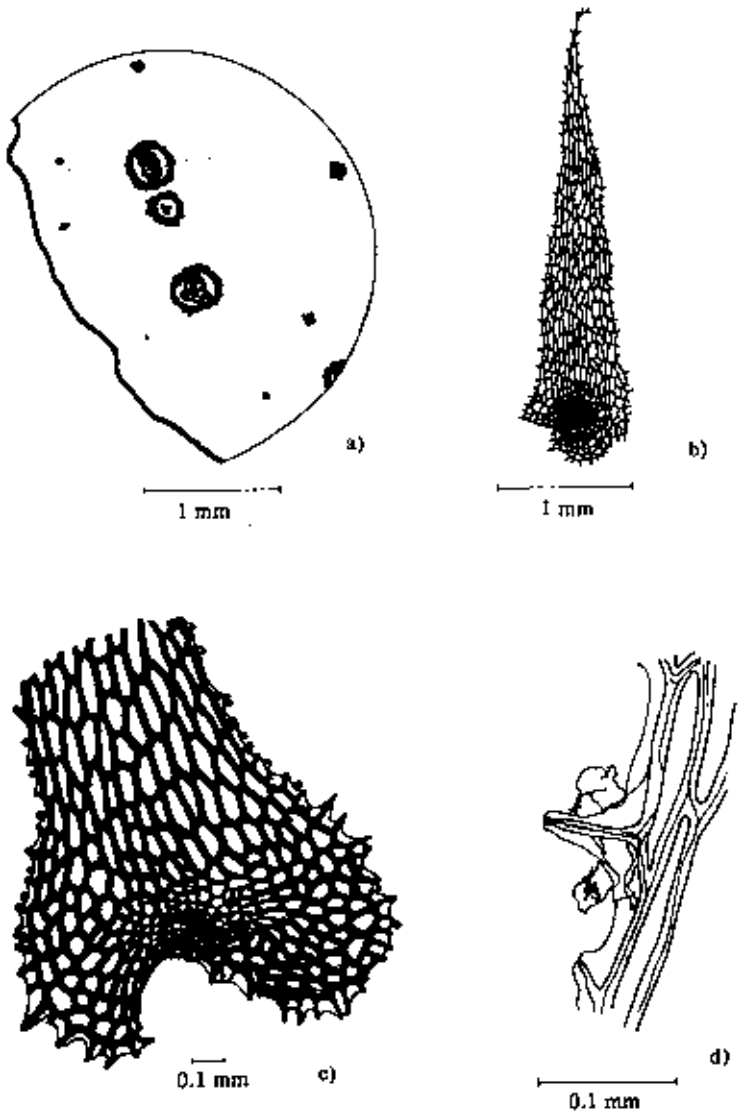

Fig. 11. Details of rhizome and rhizome scales of G. pseudoconnatum.
a) Section of rhizome cross-cut (X 40)
b) Rhizome scale (X 40)
c) Base of rhizome scale in detail (X 100)
d) Marginal trichomes in detail (X 400)

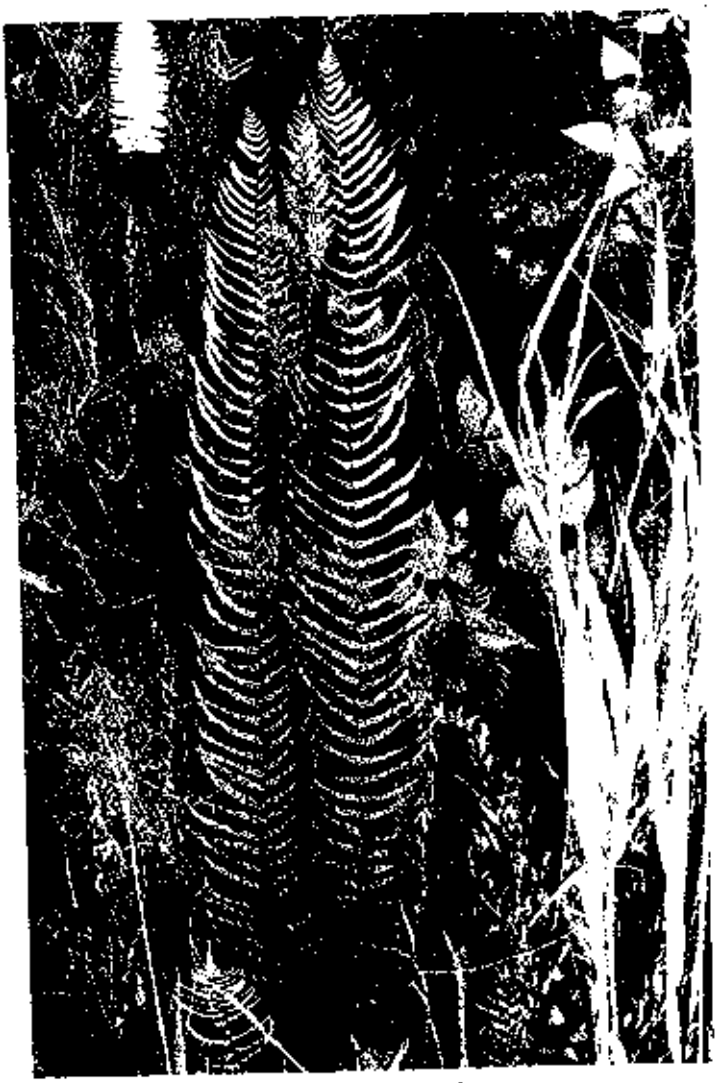

Fig. 12. Habitus of G. **subauriculatum** *(X 1[15)*

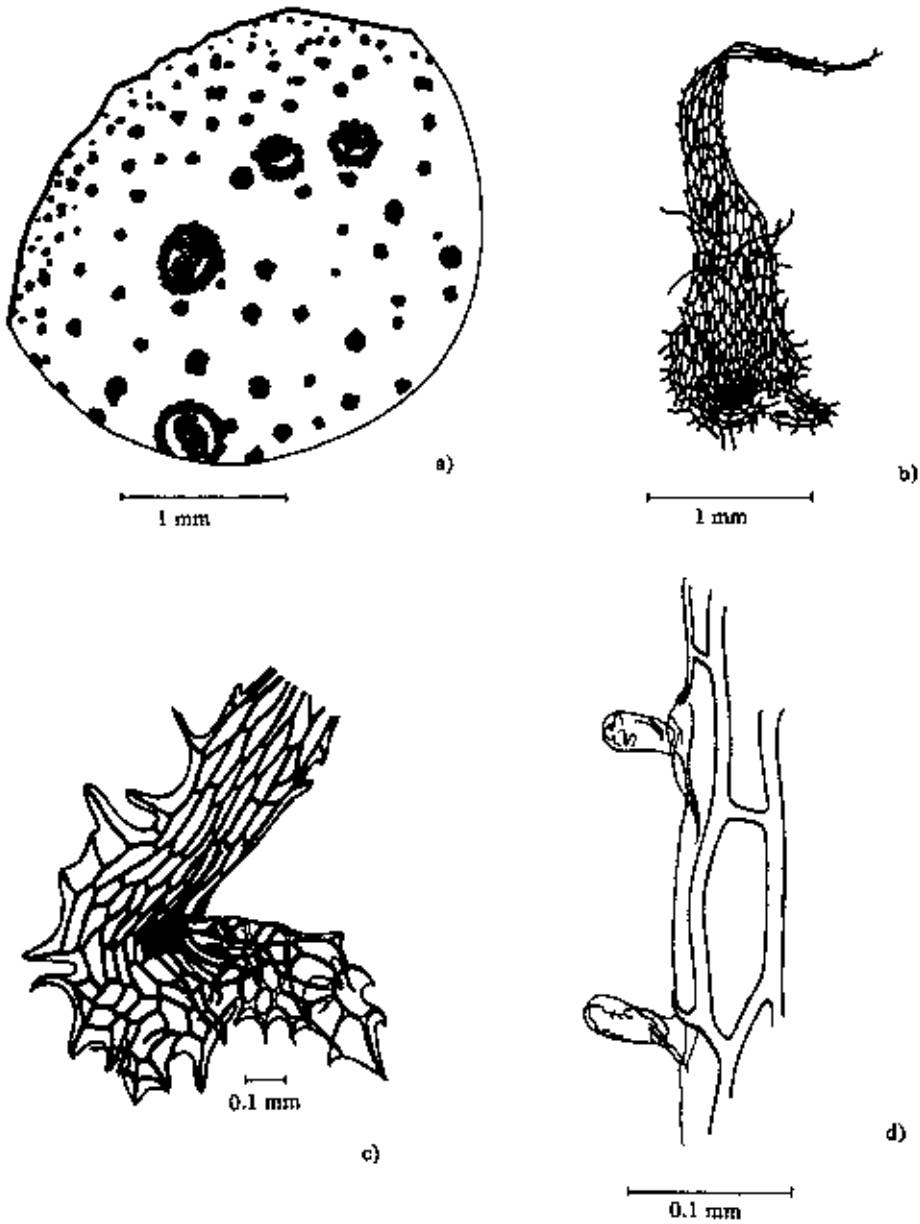

Fig. 13. Details of rhizome and rhizome scales of G. subauriculatum *(Bl.) Presl.*
a) Section of rhizome cross-cut
b) Rhizome scale
c) Base of rhizome scale in detail
d) Marginal trichomes in detail

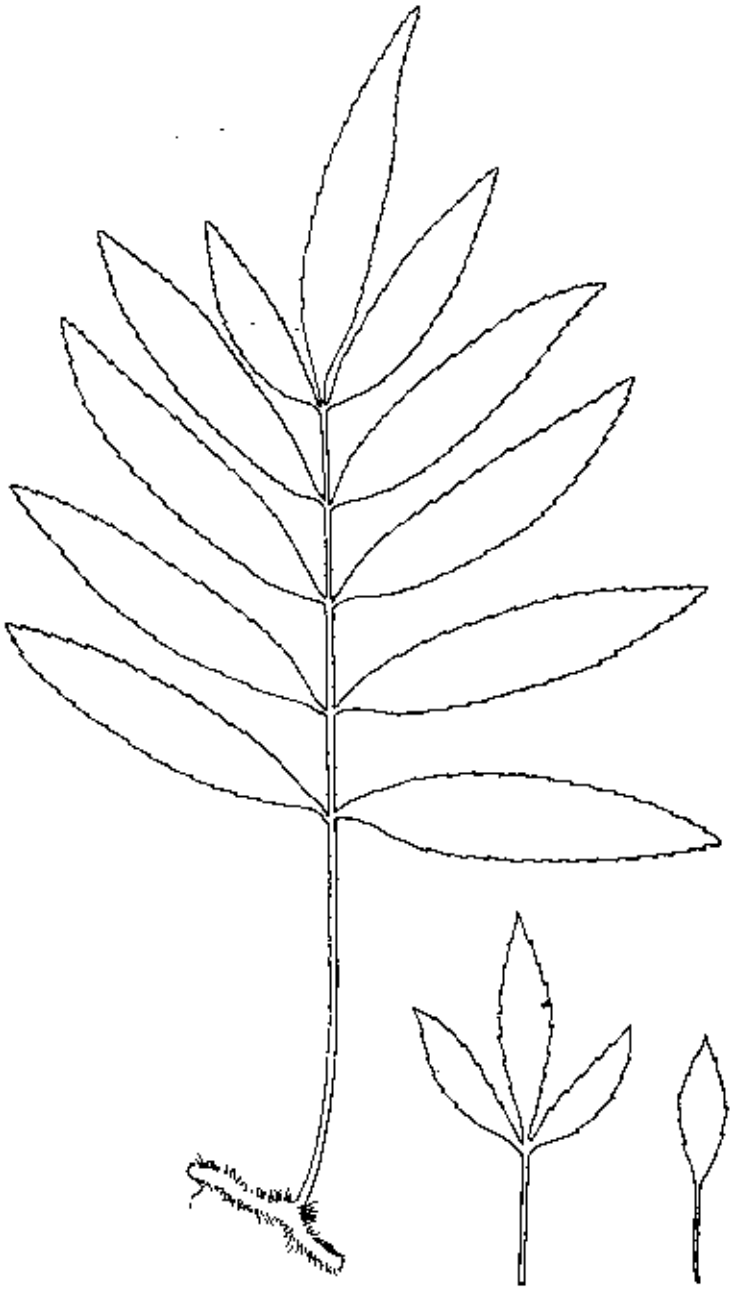

Fig. 14. Outline of G. terrestre* *(Copel)*
+ Heteroblastic leaf series

ORIGIN, DEVELOPMENT AND STRUCTURE OF THE RESIN DUCTS IN TWO PHILIPPINE *PITTOSPORUM* SPECIES

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ABSTRACT

Resin ducts in the shoot and root originate from the procambium at the shoot and root apical meristems. Schizogenous duct formation occurs in both organs, but differs considerably in the manner of development. In the shoot, a group of darkly stained cells in the procambium gives rise to the duct through the dissolution of the middle lamella and separation of cell walls. In the root, a duct-mother-cell undergoes successive divisions to give rise to the duct. The number of ducts in the shoot parallels the number of vascular bundles and appears as part of the mophological unit of the primary phloem, whereas ducts in the roots occur opposite every protoxylem pole, as well as in the phloem.

INTRODUCTION

Fernando and Zamora (1986a) found that the shoot apices of *Pittosporum resiniferum* and *P. pentandrum* show similar cytohistological zonation pattern but differ in outline, i.e., from flat to slightly convex and from concave to slightly convex, respectively. Moreover, Fernando and Zamora (1986b) claimed that the root apices of *P. resiniferum* exhibit a closed pattern of organization while those of *P. pentandrum* an open pattern of organization. The origin and development of the primary tissues of the root and shoot of the two species were described in the foregoing papers.

This article, which is the third in the series, describes the origin, development and structure of the resin ducts in the shoot and root of *P. resiniferum* and *P. pentandrum*.

MATERIALS AND METHODS

Stems, shoot and root apices of *P. resiniferum* were collected from the experimental plots of the Forest Research Institute in Baguio City and Bureau of Plant Industry in Los Baños, Laguna, as well as from plants growing in the wild in Mt. Sto. Tomas, Baguio City. For *P. pentandrum*, samples were gathered from plants growing on the campus of the University of the Philippines at Los Baños, Laguna and Guimaras Island, Iloilo. The materials were fixed in formalin-aceto-alcohol, dehydrated through the tertiary butyl alcohol series (Johansen, 1940) and embedded in Tissue Mat. Serial longitudinal and transverse sections were cut at a thickness of 6-8 microns and stained with safranin-fast green or safranin-aniline blue. Histochemical test for resin was performed by soaking fresh, thin sections of stems, leaves and roots in 7% aqueous cupric acetate for several days, until a greenish copper precipitate occurs.

The manner of differentiation of the duct was followed through serial transverse sections from the region of the initials upwards and downwards of the root and shoot, respectively.

RESULTS AND OBSERVATIONS

A. *Resin Duct in the Shoot*

The stems, petioles and leaves of *P. resiniferum* and *P. pentandrum* possess resin ducts that arise from the procambium at the shoot apical meristem and share parallel development with the vascular tissues. In fact, transverse sections of these organs show that the number of resin ducts more or less parallels the number of vascular bundles (Figs. 1, 2 and 3), although the resin ducts are formed earlier than the vascular tissues. Figure 4 shows the resin duct as part of the morphological unit of the primary phloem.

The resin ducts develop schizogenously through the dissolution of the middle lamella and separation of cell walls of a group of loosely arranged, darkly stained cells at the procambium (Fig. 5). The duct increases in size through the further dissolution of the middle lamella and separation of the walls of some more cells which are duct initials. The duct later on develops separately from the procambium some distance from the shoot apical meristem (Fig. 6). The cells lining the duct would then specialize as resin duct cells and functionally secrete resin. In transverse sections, about 10-20 resin duct cells surrounding a cavity make-up the mature resin ducts of the stem, petiole and leaf.

In longitudinal sections, the resin ducts run parallel to the axes of the stem, petiole and leaf. The resin duct cells are narrowly-elongated, characterized by having thin-walled, uninucleated parenchyma cells which stained more densely than the surrounding cells. Numerous granule-like particles were observed inside each resin duct cell (Fig. 7).

B. *Resin Duct of the Root*

Resin ducts occur in the roots of both species of *Pittosporum* and these originate from the procambium of the root apical meristem. They develop earlier than the vascular tissues of the root. In transverse sections, they occur opposite every protoxylem pole as well as in the phloem. However, at a stage in the ontogeny of the root where vacuolation of cells in the vascular cylinder becomes extensive, those resin ducts which lack secretion are difficult to identify (Fig. 8).

The development of the resin ducts in the roots of both species is schizogenous which involves: (1) two successive divisions (tangential and oblique, respectively) of a duct-mother-cell to form four sister cells (Figs 9 and 10); (2) development of a small cavity at the junction of the tangential and newly formed oblique walls as a result of the dissolution of the middle lamella and, consequently, separation of the cell walls which in transverse sections form a quadrangular outline (Figs. 11 and 12); and (3) further increases in size of the duct through the divisions of the initial four cells in a plane perpendicular to the surface of the duct (Fig. 13). These cells lining the duct form the resin duct cells and function to secrete resin. In transverse sections, mature ducts of the root are composed of 5-10 resin duct cells.

In longitudinal sections, the resin ducts of the roots run parallel to the axis of the root. The resin duct cells are broadly elongated, thin-walled, uninucleated with large nucleus and prominent nucleoli. They are somewhat similar in size to those in the adjacent cells but stained more densely.

DISCUSSION

Allen (1947) referred to the secretory cells which are associated with the procambium as stelar secretory cells while Spurr (1950) termed them vascular secretory cells. Tetley (1925) suggested that the term "secretory canal" seems preferable until more information is known about the chemistry of the canal content. In *Pittosporum*, several researchers (Bacon, 1909; Bakker and Van Steenis, 1957; Metcalfe and Chalk, 1957; Jay, 1969; Noble, 1978) reported the presence of a resinous substance in all the organs examined and is considered here as due to the ducts, hence, the term "resin duct". A confirmatory test for resin proved positive, i.e., greenish copper precipitate was observed from sections of stems, leaves and roots treated with 7% aqueous cupric acetate.

Although the resin ducts of the shoot and root originate schizogenously from the same group of initials at their respective apices (i.e., procambium), their development differs considerably. In the shoot, a duct is formed from a group of darkly stained cells, through the dissolution of the middle lamella and separation of cell walls. In the root, the duct originates from successive divisions of a duct-mother-cell. Esau (1965) pointed out that the separation of cells in the formation of schizogenous secretory space may occur with or without preceding division of cells.

The first appearance of the ducts in the shoot and root was in the region immediately behind the apical meristem of both apices. The ducts could be observed before there is any trace of differentiating vascular elements. In the root, the duct-mother-cell can be distinguished in the region where the procambium differentiates or even earlier. The duct-mother-cells are larger than the procambial cells adjacent to them. The significance of the invariable occurrence of the ducts opposite the first differentiating vascular element is that the original contents of the ducts are regarded as being fatty in nature, most of which are derived from the developing phloem elements (Tetley, 1925). Williams (1954) claimed that the ducts serve to supplement the sieve tubes in the conduction of organic nutrient material into the growing apices. Werker and Fahn (1969) found that the number of resin ducts in the primary body of the root corresponds to the number of xylem strands. In *Pittosporum*, same correlation was observed in all the organs examined.

In the shoot, the resin ducts appear as a part of the morphological unit of the primary phloem, while in the root, the ducts occur opposite every protoxylem pole, as well as in the phloem. Certain Coniferae also show a characteristic distribution of the resin duct in the primary vascular region of the seedling taproot (Guttenberg, 1943). In Araucariaceae, the ducts occur in the primary phloem, while in the Pinaceae, a single central duct or one duct is associated in each protoxylem pole.

Based on the sections made on stems, petioles, leaves and roots, all these organs possess resin ducts. In terms of diameter, those of the root are the smallest while those of the stem are the biggest. The diameter of the ducts in the petiole and leaf possesses intermediate values. Tetley (1925) pointed out that the increase in the size of the canal is accompanied by a corresponding increase in the amount of its contents. A colorless or yellow-gumlike content of the resin duct of the shoot and root is observed in both species of *Pittosporum*. This had also been observed by Metcalfe and Chalk (1957) for *P. tenuifolium*. The only reported function of the duct is the secretion of a resinous substance which Bacon (1909) and Noble (1978) identified as containing dihydroterpene and normal-heptane, an essential component of gasoline.

There was a direct relationship observed between the diameter of the plant organs and the number and diameter of the resin ducts. *Pittosporum resiniferum* has more robust shoots than *P. pentandrum*. Thus, this species has more and larger ducts. In this regard, *P. resiniferum* could be considered economically more important than *P. pentandrum*.

A study of the fine structure of the resin duct cells is necessary to verify the probable relationship between morphological specialization and resin synthesis. A cytohistochemical study of the whole plant at primary and secondary stages of growth could confirm the distribution and extent of the resinous substance in the various organs and possibly could shed light on the presence of other important substances present in the plant.

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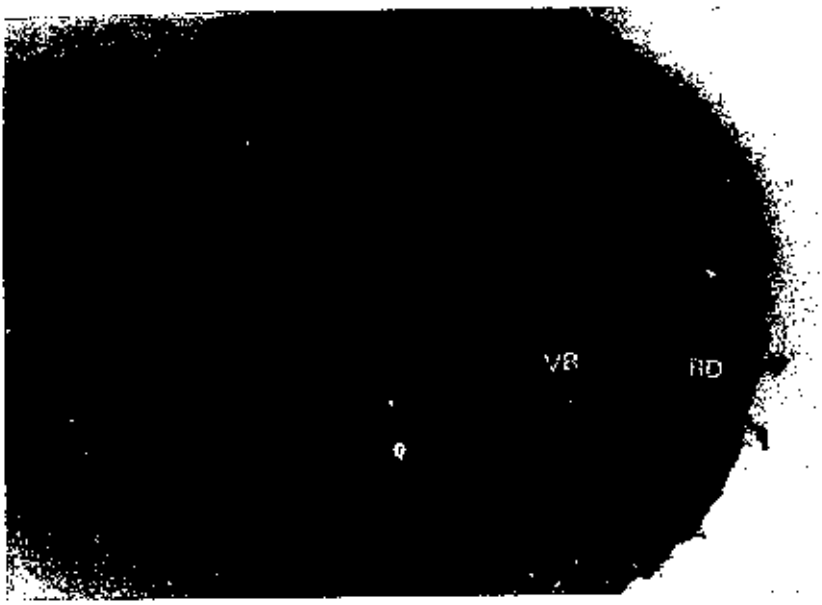

Fig. 1. Photomicrograph of a transverse section of a young stem of Pittosporum pentandrum *showing vascular bundles (VB) and resin ducts (RD) (X 611).*

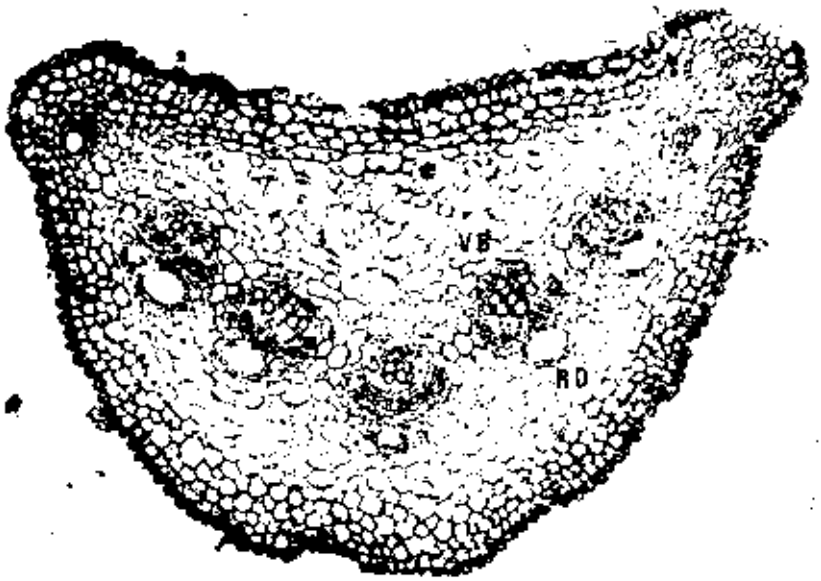

Fig. 2. Photomicrograph of a transverse section of a young petiole of Pittosporum resiniferum *showing vascular bundles and resin ducts (X 800).*

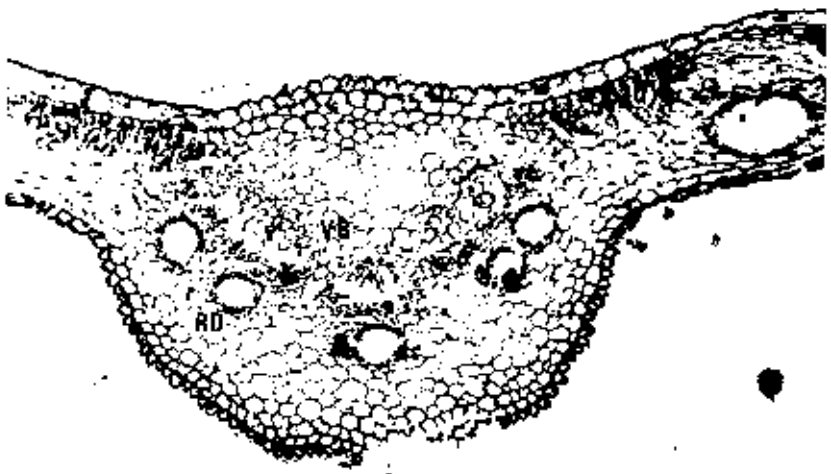

Fig. 3. Photomicrograph of a transverse section of a young leaf of P. resiniferum *showing vascular bundles and resin ducts (X 800).*

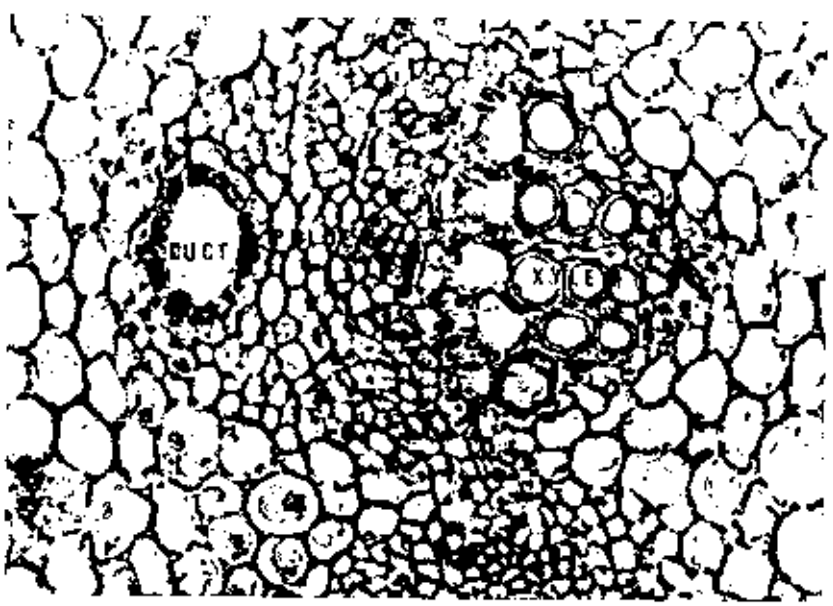

Fig. 4. Photomicrograph of a portion of a transverse section of a stem of P. resiniferum *showing the duct as part of the morphological unit of the primary phloem (X 2200).*

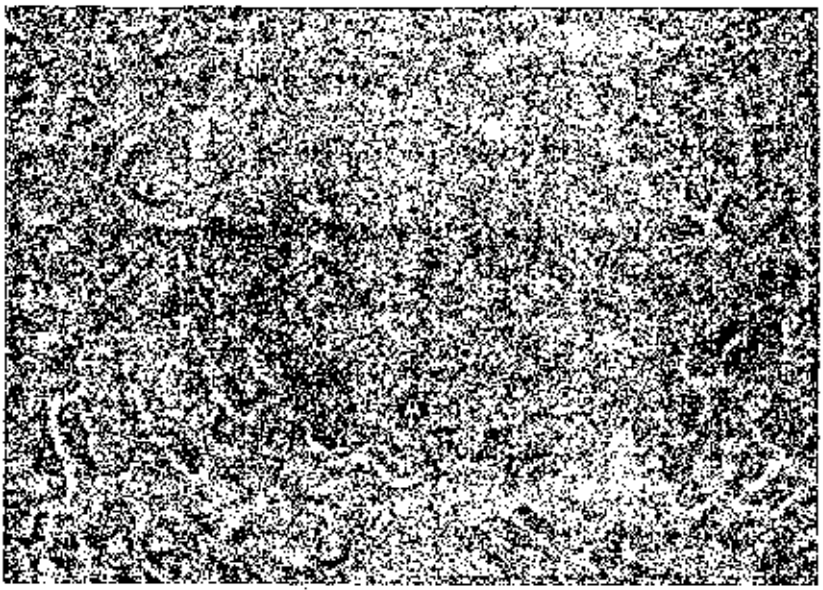

Fig. 5. Photomicrograph of a transverse section of a vegetative shoot of P. resiniferum *showing densely stained group of irregularly arranged cells (X 1150).*

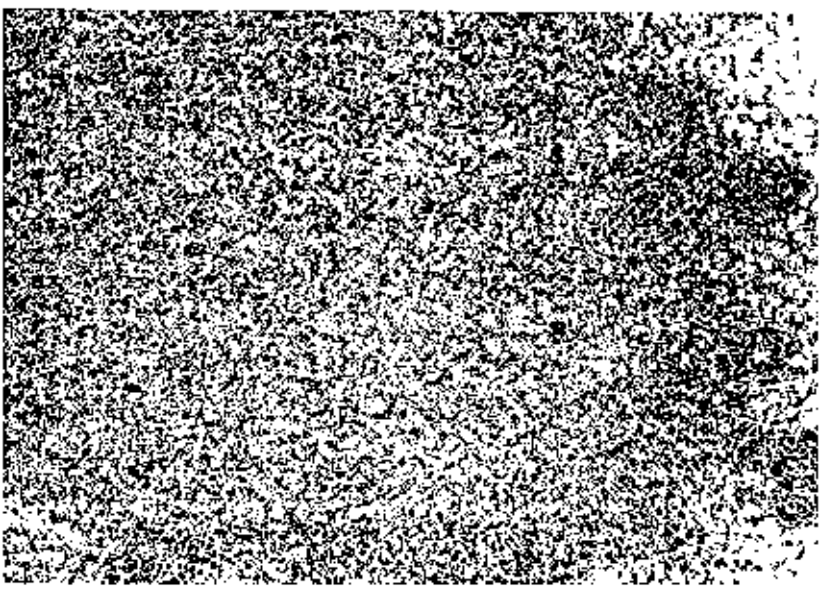

Fig. 6. Photomicrograph of a transverse section of a vegetative shoot of P. resiniferum *showing slightly developed resin duct and darkly stained procambium (X 1150).*

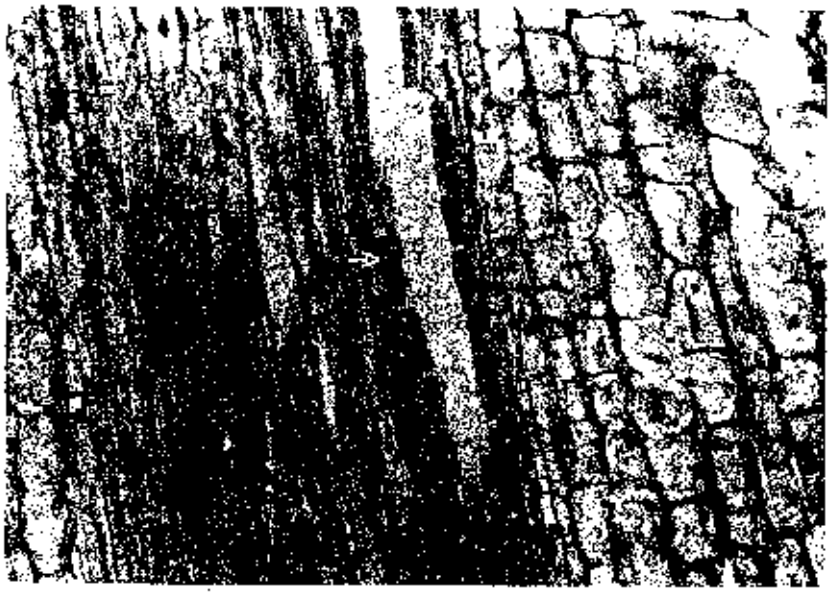

Fig. 7. Photomicrograph of a portion of a longi-section of a stem of P. resiniferum *showing the duct in longitudinal view (X 1666).*

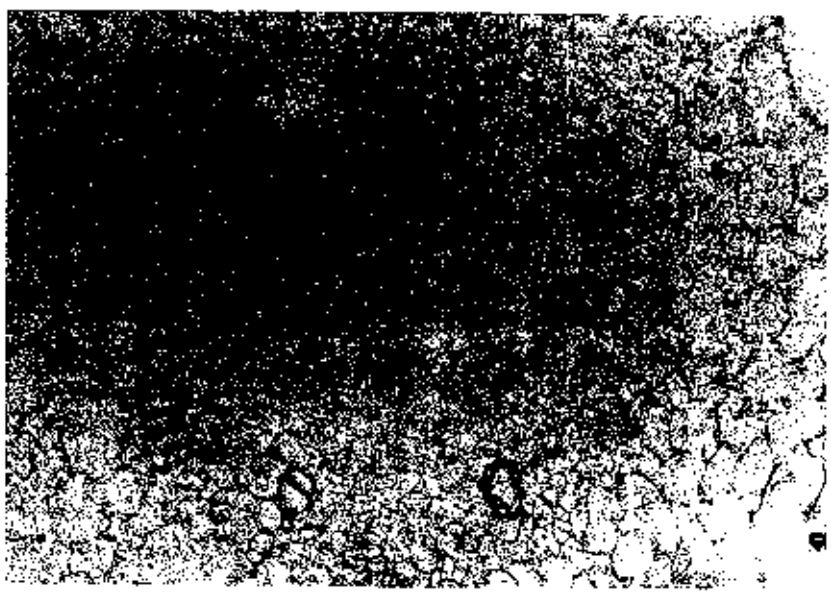

Fig. 8. Photomicrograph of a portion of a transverse section of a root of P. pentandrum *showing 5 alternating phloem and xylem poles (X 2125).*

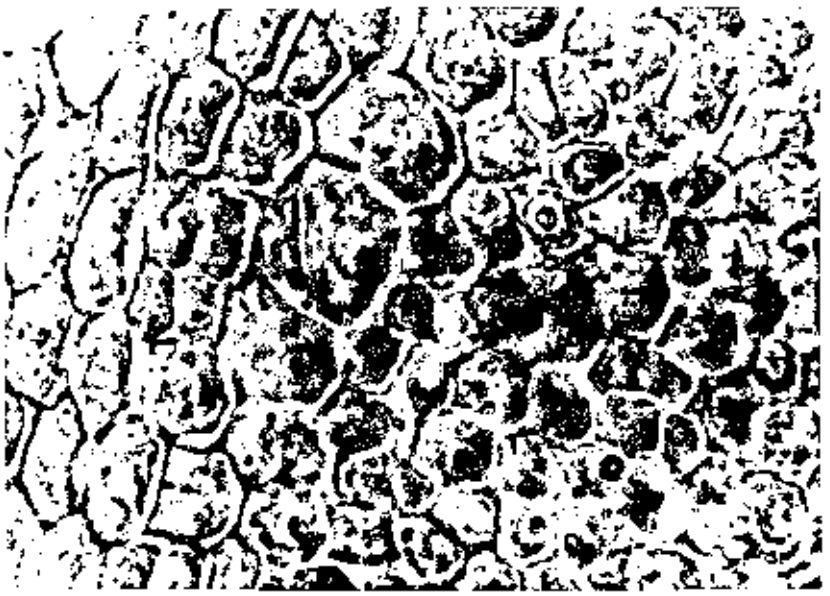

Fig. 9. Photomicrograph of a portion of a transverse section of a root of P. resiniferum *showing a duct-mother-cell after having undergone a tangential division (X 1785).*

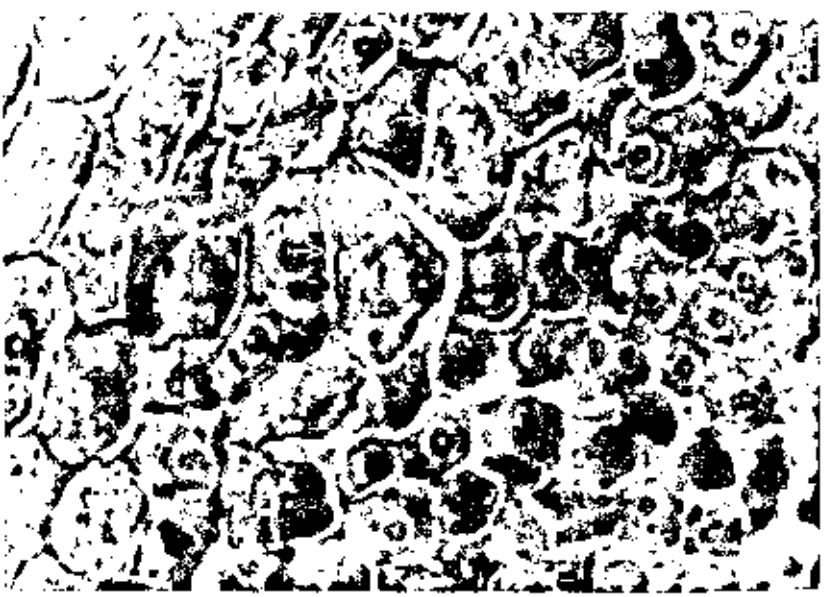

Fig. 10. Photomicrograph of a portion of a transverse section of a root of P. resiniferum *showing the duct-mother-cell after having undergone oblique division, just after the tangential division (X 1785).*

Fig. 11. Photomicrograph of a portion of a transverse section of a root of P. resiniferum *showing a cavity arising at the junction of the newly formed tangential and oblique walls (X 1785).*

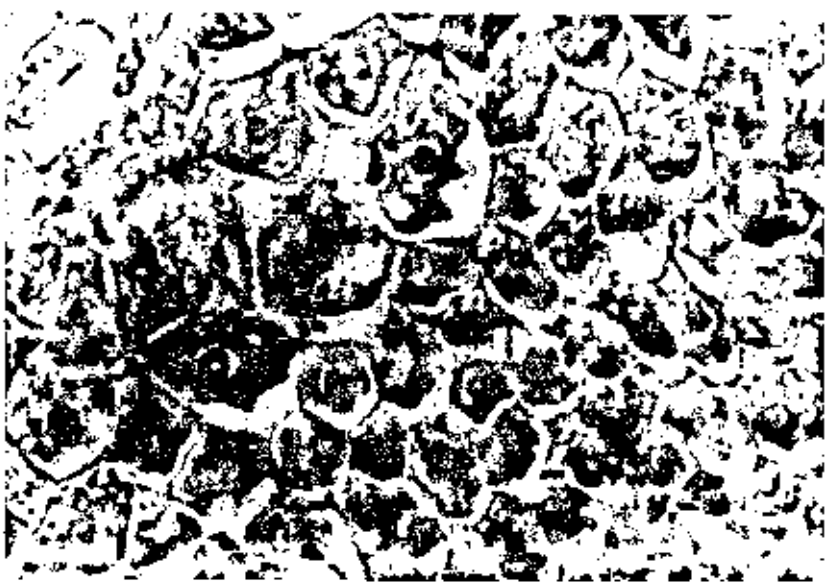

Fig. 12. Photomicrograph of a portion of a transverse section of a root of P. resiniferum *showing the duct increase in diameter through the continuous dissolution of the middle lamella and further dissolution of the walls (X 1785).*

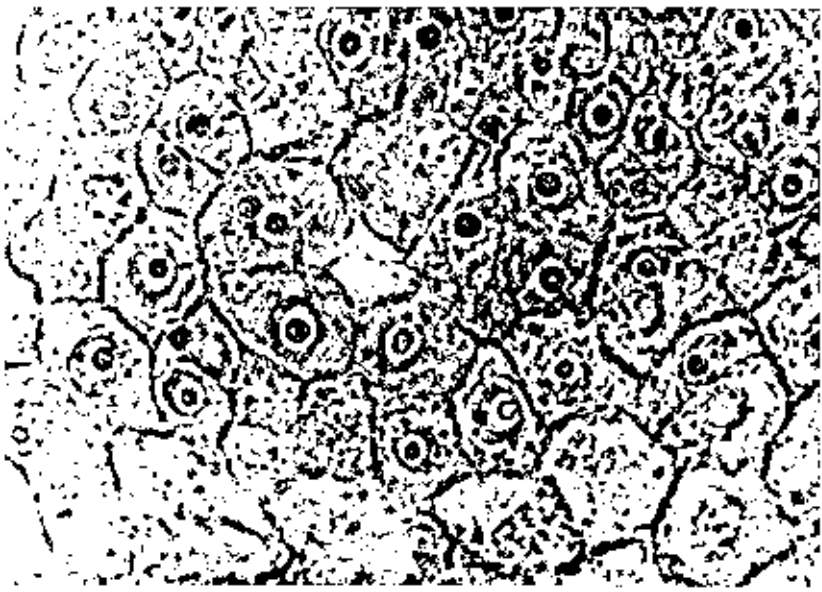

Fig. 13. Photomicrograph of a portion of a transverse section of a root of P. resiniferum *showing the duct further increase in diameter through the division of the initial four cells in a plane perpendicular to the surface of the duct (X 1875).*

Fig. 14. Photomicrograph of a portion of longi-section of a root of P. resiniferum *showing the duct in longitudinal view (X 1666).*

ISOLATION AND SCREENING OF FUNGI STRAINS WITH GIBBERELLIN-LIKE ACTIVITIES

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ABSTRACT

Diseased plants belonging to the Gramineae family were collected and fungi showing the characteristics of *Fusarium* and *Gibberella* were isolated. Out of the 30 isolates, seven (7) were found to produce stimulatory activities. Further screening gave one very good strain which showed consistently high gibberellin-like activity by both plant bioassay and microbiological assay.

INTRODUCTION

The gibberellins are plant growth hormones produced by *Fusarium moniliforme* Sheld. and the perfect stage *Gibberella fujikuroi* (SAW). These metabolic products have found wide application in agriculture by enhancing growth of trees and field crops, improving grain quality, promoting seed production, inducing flowering, increasing the size of fruits, etc.

The growth-promoting activity of the gibberellins was first discovered by Kurosawa (1926) in Japan when he applied cell-free filtrates from a pure culture of the fungus to rice seedlings and obtained enhanced growth. Work was extended to other plants by other Japanese investigators followed by American and European researchers. Wittwer and Bukovac (1957) found that soaking seeds in gibberellic acid accelerated the germination of peas, beans and other plants. Lange (1957) observed that a number of tropical plants grown in Hawaii responded to weekly applications of gibberellin. Young seedlings of papaya, guava, coffee, banana and citrus increased in growth by 35% and 80% in height at concentrations of 10 and 100 ppm of gibberellin. Rappaport *et al* (1959) reported that when Burbank potatoes were dipped in 25 ppm of gibberellic acid for one and a half hours, 90 per cent of the seeds sprouted in one month compared with only 11 per cent of the untreated ones.

In the Philippines, Magsino (1958) reported the use of gibberellic acid on garlic in commercial scale. Limited studies had been undertaken due to the exorbitant price and difficulty of obtaining the chemical from abroad. Research and development activities in the production of the plant growth hormone therefore should be done in connection with the national program of increased food production.

MATERIALS AND METHODS

Collection of diseased plants. – Samples consisting of diseased palay, corn and sugar cane plants collected from different places in Batangas, Laguna, La Union, Baguio and Pampanga provinces were used as sources of gibberellic acid-producing strains of *Fusarium*.

Isolation of mold. – Isolation was done by the pour-plate dilution method on Czapek's Dox Agar (CZDA), a modification of the Czapek formula of Dox prepared by Thom and Raper (1945). Microscopic examinations were undertaken to identify *Fusarium* strains characterized by their typical crescent-shaped or spindle-shaped macroconidia and round, egg-shaped or pear-shaped microconidia. These strains were purified by repeated platings and transferred on Czapek's Dox Agar slants at room temperature.

Screening of isolates for their gibberellin-like activities. The growth stimulatory responses of the selected isolates were determined using the modified process of Gordon's (1960) plant bioassay and the rapid microbiological assay method at Barea *et al* (1974). Liquid cultures of the selected isolates including a known species of *F. moniliforme* 5509 obtained from Thailand Institute of Science and Technology Research (TISTR) were used. Gibberellic acid crystals used were obtained from Beckman Laboratory, Anaheim, California.

a) Plant Bio-Assay Method:

Seeds Used. Seeds of forty-one dwarf varieties of corn, rice, and sorghum were obtained thru the courtesy of Mr. Emiliano P. Gianzon, Assistant Director for Research of the Bureau of Plant Industry and International Rice Research Institute. The seeds were those of the most diseased and insect resistant varieties in Asia especially the rice variety, IR-36.

Preparation of seeds. Seeds were sterilized by soaking in one ppm mercuric chloride solution in sterile distilled water for five minutes, then washing several times with sterile distilled water.

Preparation of extract of fungi strains. Fungal isolates were grown in Czapek's Dox Agar slants for 10 days at room temperature. After 10 days the culture was scraped and transferred into 100 mL sterile distilled water with 2 drops of Tween 20 and thoroughly mixed in a cycle mixer. After five hours, the supernatant liquid was decanted into sterile vials to be used in the tests.

Four dwarf seeds were placed on sterile moist filter paper in a sterile Petri dish, covered and allowed to germinate for 72 hours. Two drops of the culture filtrates of each of the *Fusarium* isolates were applied to the leaf axils of the 72-hour seedlings by means of a medicine dropper twice daily for 3 days. Seedlings were allowed to grow and observations on growth were taken after 4 days to two weeks. All measurements (in mm) were made from the axils to the tip of the leaf.

Pure crystals of gibberellic acid were used as control in various concentrations ranging from 2 to 10 ppm at 2 ppm interval and applied as above.

b) Microbiological Assay Method

Yeast cultures used. Fifteen different yeast isolates obtained from the Microbiological Culture Collection of the National Institute of Science and Technology (NIST) were used in the assay. These yeast cultures were maintained in nutrient basal NG agar medium developed in 1958 by Lindergren, Nogai and Nogai.

Preparation of yeast inoculum. A 24-hour-old culture of each isolate grown in NG agar medium at room temperature was used to prepare the inoculum. The yeast culture was inoculated into 50 mL NG liquid medium in a 250 mL Erlenmeyer flask and incubated on a rotary shaker (140 rpm) at room temperature for 24 hours. One per cent of the inoculum was used for inoculating each of the culture flasks in the succeeding experiments.

Preparation of the inoculum of Fusarium strains. A 10-day old culture of each isolate grown at room temperature in CZDA slant was used to prepare the inoculum. The culture was scraped and transferred into 100 mL sterile distilled water with 2 drops of surfactant, (Gordon, 1960) and thoroughly mixed in a cycle mixer. The suspension was filtered through glass wool to obtain a homogeneous suspension of conidia. Inocula used were 5, 10, 15 and 20 per cent.

Assay method. Coconut water (CW) which is a good microbial medium as reported by Palo and Lapuz (1954) was incorporated in NG liquid medium. Three batches of the coconut water in NG (CWNG) liquid medium at different CW concentrations (10, 25, and 50%) were prepared and distributed in volumes of 50 mL in 250 mL Erlenmeyer flasks. After steriziling and cooling the medium to room temperature each batch was inoculated with one per cent of the prepared yeast inoculum simultaneously with each of the different concentrations of the prepared *Fusarium* inoculum and incubated on a rotary shaker (140 rpm) at room temperature for 24 hours.

Optical density measurements at 660 nm of all samples were made after 24 hours.

Preparation of standard curve of gibberellic acid for the microbiological assay. Different concentrations of pure crystals of gibberellic acid (5, 10, 15, and 20 ppm) were prepared. One mL each of the GA solution was added to 50 mL CWNG liquid medium with different CW concentrations contained in 250 mL Erlenmeyer flasks. The flasks were inoculated with one per cent yeast inoculum and then incubated on a rotary shaker (140 rpm) at room temperature for 24 hours.

Optical density measurement at 660 nm of all samples were taken after 24 hours.

Confirmatory test for the presence of acid. To detect the presence of acid in the selected *Fusarium* isolates that responded significantly to both assays, the mycelium with conidia of the 10-day-old culture of each isolate was streaked into CZDA medium with

3% calcium carbonate in sterile Petri dishes and incubated at room temperature. The presence of acid shows a dark zone around the colonies after 14 days due to the dissolution of $CaCO_3$.

Characterization of isolate F-7. Morphological and cultural characteristics of F-7 were studied microscopically and its identity determined according to Alexopoulus (1962), Von ARx (1974), Clements (1957), Funder (1953) and Wolf (1947), Wollenweber and Reinking (1925).

RESULTS AND DISCUSSION

From 300 diseased plants belonging to the Gramineae family composed of palay, corn and sugar cane, 30 *Fusarium*-like species characterized by their typical spindle-shaped macroconidia were isolated.

Screening tests showed that 7 *Fusarium* strains produced stimulatory effect on the plants as well as the known species of *Fusarium moniliforme*. However, only one isolate, F-7, from a diseased corn, showed stimulatory activities in all the assays used.

As shown in Table 1, it is noticeable that after seven days, only F-7 produced the most significant response in all the different host dwarf plant seedlings. The rice and corn seedlings were stimulated by almost all the selected strains particularly F-7, attaining a maximum mean leaf length of 197.5 mm in corn EG-SYN 106 as compared to 60 mm in the untreated seedlings as shown in Plate I, Fig. 1. This was followed by F-29 which stimulated rice IR-36, giving the highest measurement of leaf length of 119 mm compared to 88 mm in untreated seedlings after a period of one week. It was further observed that the leaves of the stimulated seedlings were paler green in color than the untreated ones which corroborated with the findings of Marth (1956). Except for Sorghum BPI-PB-2, all other host dwarf seedlings were not stimulated by *F. moniliforme* 5509. All increases in leaf length measurements of treated seedlings were significant.

In Table 2, it was observed that a concentration of 10 ppm gibberellic acid favored leaf growth of rice and corn seedlings after 7 days as shown in Plate I, Fig. 2. One of the pronounced effects of gibberellic acid is the rapid lengthening of the stems and internodes of plants. Yabuta and Hayasi (1939), Brian *et al* (1954), Wittwer and Bukovac (1957) reported that soaking of seeds in gibberellic acid accelerated the germination and development of seedlings of beans, peas and other plants. Spraying 2 to 1000 ppm concentrations of gibberellic acid proved effective in hastening flowering and development of seedless fruits. No further attempts using more than 10 ppm gibberellic acid were tried on the host plant seedlings.

The microbiological growth assay using yeast showed the effect on the two yeasts, *Saccharomyces cerevisiae var. ellipsoideus* (BAW_2) and *Saccharomyces pastorianus* Y-IV of the different concentrations of cell filtrates of the selected *Fusarium* strains. Of the yeast strains used in the assay, BAW_2 yeast gave the most consistent results. As shown in Figure 1, concentrations of 5, 10, 15, and 20 per cent of the F-7 cell filtrate in 10 and

25% CW concentrations in NG liquid medium using BAW_2 and Y-IV yeasts indicated a linear increase in optical density following Beer's Law. It is noticeable that as the concentration of the cell filtrate and coconut water mixture increases, the optical density increases. Not much is known about the influence of these growth stimulatory substance on the growth and reproduction of microorganisms but in some cases they stimulate microbial growth, according to Barea *et al* (1973). Lu *et al* (1958) reported that treating soil with gibberellic acid increased the number of *Azotobacter* cells. Hormones applied as foliar sprays increased the rhizosphere microflora of leguminous plants. Sullia (1968) and Anilkumar and Chakravarti (1970) showed that treatment of roots of maize seedlings with gibberellic acid at 1 ppm stimulated growth of fungi and bacteria in the rhizosphere.

As shown in Figure 2 the higher the coconut water concentration in NG liquid medium the higher the optical density. However, BAW_2 yeast further indicates it's efficiency as test organism in 10% CW in NG medium by showing an increase in different gibberellic acid concentrations after 24 hours.

The high optical density produced by F-7 strain using the microbiological assay method could be an index of the growth stimulatory activity which confirms the results obtained in the plant growth assay.

Confirmatory test for gibberellic acid content of F-7. In plates of CZDA agar medium with 3% calcium carbonate, the F-7 strain produced a dark zone around the colonies after 14 days as shown in Plate 2, Figure 4, indicating formation of acid which caused the dissolution of the fine white calcium carbonate powder.

The gibberellic acid mold identity. Identification was based on the cultural and morphological characteristics exhibited by the selected F-7 isolate when grown on potato dextrose and Czapek's dox agar media.

Due to the great variability of this strain in the two media, difficulties were encountered in the identification so it was compared with the known *Fusarium moniliforme* 5509 strain used in this study.

CULTURAL CHARACTERISTICS

Agar colonies. On Czapek's Dox agar, hyphae of F-7 and *Fusarium moniliforme* 5509 became visible after 48 hours. On the 7th day the mycelial growth of the two strains were fully developed which was creamy in color. The cottony mycelium of F-7 became violet on the underside of the agar slants after one month and as the culture aged it became dark violet. The mycelia of F. moniliforme 5509 appeared to be loose, radiate and change in color from creamy to violet after 12 days.

On potato dextrose agar, the two strains showed the same characteristics as those produced on Czapek's Dox agar except that the known 5509 strain sporulated quickly covering the entire nutrient agar after four days.

MORPHOLOGICAL CHARACTERISTICS

In both media, F-7 differs from the known 5509 strain by having erumpent and clustered gibberellic-like microconidia, mostly in chains formed by aerial mycelium (Plate 3, Fig. 5). The microconidia of the known strain are not borne in chains. After three weeks, F-7 showed abundant microconidia appearing at the tips and sides of the hyphae with spindle and sickled-shaped macroconidia (Plate 3, Fig. 6). Absence of chlamydospores was observed. Wollenweber (1925) stressed that in typical *Fusarium moniliforme* strains, the culture which are violet on the underside of the tube agar are easily distinguishable by the absence of chlamydospores and by the chainlike arrangement of the microconidia. Perithecia of F-7 and *Fusarium moniliforme* 5509 have not been found in all the microscopic examinations. However, Synder and Hansen (1945) reported that perithecia of *G. fujikuroi*, the perfect stage of *F. moniliforme* are expected to occur particularly during the coldest season of the year. Several authors stated that some perithecia of the perfect stage of *Fusarium moniliforme* will not be produced if the strain is bisexual and self-fertile. These reports may be attributed to the absence of perithecia in both strains. It was also observed that the temperature has an appreciable effect on the morphology of conidia as well as the rate of growth of the mycelium and may also affect color intensity. At higher temperatures of 31°C, conidia of both strains appear to be slender and attenuated after 4 weeks. Conidia of F-7 became thinner with pionnotes as shown in (Plate 4, Fig. 7) while at 27°C they appear to be ovoid, fusoid and globose.

Based on the cultural and morphological characteristics, slight differences were observed between F-7 and known 5509 strain. Inspite of the absence of perithecia which may be due to high temperature, the behavior of F-7 strain resembles *Fusarium moniliforme* Sheld. *var erumpen n. var* species under section *Liseola* as described by Wollenweber and Reinking (1925).

SUMMARY AND CONCLUSION

1. Of the pure cultures of *Fusarium* strains isolated, 7 were found to produce stimulatory activities.

2. Using the plant and microbiological growth assays, F-7 showed the most significant response.

3. The standard solution of gibberellic acid crystals and the fungal filtrate of F-7 in different concentrations showed comparative activities in the plant and microbiological assays.

4. Of the 15 yeast strains tested, *Saccharomyces cerevisiae var ellipsoideus* (BAW_2) yeast was found to be the best test organism in the microbiological growth assay.

5. Morphological and cultured characteristics of the local isolate F-7 resemble *Fusarium moniliforme* Sheld. *var erumpens n. var* species under Section *Liseola* of the Genus *Fusarium* described by Wollenweber and Reinking (1925).

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Table 1. Effect of the cell filtrate of selected *Fusarium* strains on the leaf length of different host plants after seven days.

Fusarium Strain:	Leaf Length (mm)											
Isolate Number	Rice				Corn				Sorghum			
	Control	IR-36	Control	IR-38	Control	EG Syn # 106	Control	EG Syn # 109	Control	BP1 PB-2	Control	CS 108
F – 4	53.0	90.0	52.3	55.0	81.0	82.1	88.0	90.1	98.0	96.0	102.0	98.2
F – 29	88.0	119.0	75.0	101.0	89.0	97.0	80.0	89.0	89.0	90.4	112.0	101.0
F – 7	72.0	114.4	86.0	115.1	60.0	193.5	72.3	114.0	93.0	105.0	101.0	196.0
F – 20	90.2	98.5	96.0	101.2	92.1	95.2	101.0	105.0	92.0	88.1	110.0	111.0
F – 5509	64.1	75.1	55.0	65.0	100.0	101.5	86.0	88.0	89.0	186.5	160.0	175.0

Table 2. Effect of different gibberellic acid concentrations on the leaf length of different host plants after seven days.

Gibberellic Acid	Leaf Length (mm)											
	Rice				Corn				Sorghum			
Concentration ppm	Control	IR-36	Control	IR-38	Control	EG Syn #106	Control	EG Syn #109	Control	BPI PB-2	Control	CS 108
2	55.0	56.2	60.0	61.0	99.1	102.0	95.0	96.5	99.0	98.2	104.0	101.5
6	88.0	88.1	91.0	95.0	84.0	86.5	81.1	125.0	90.1	92.4	105.0	104.1
8	91.0	92.0	99.0	104.0	90.2	108.0	88.0	128.0	93.2	100.0	102.0	150.0
10	65.0	169.5	90.0	160.0	71.0	125.0	88.1	139.0	90.2	100.0	149.0	152.0

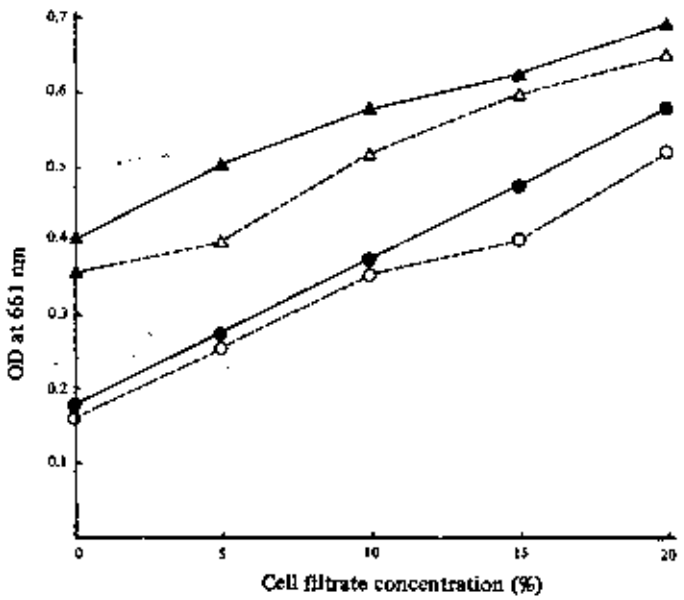

Fig. 1. Effect of different filtrate concentrations of F-7 on the yeasts, S. ellipsoideus, *BAW_2 and* S. pastorianus, *Y-IV with different CW concentrations in NG liquid medium; 1% BAW_2 + 10% CW (–•–), 25% CW –▲–), 1% Y-IV + 10% CW (– – –o– – –), 25% CW (– – –△– – –) after 24 hours.*

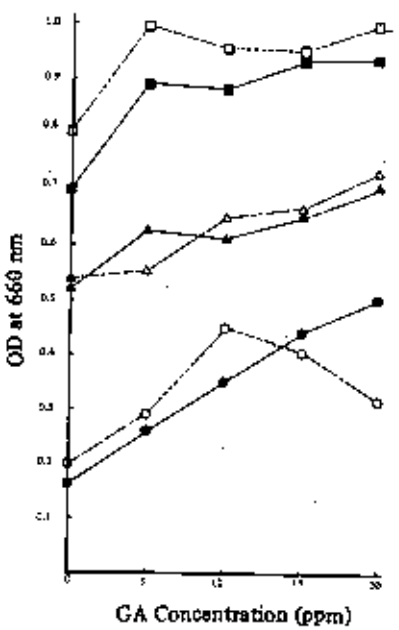

Fig. 2. Effect of different GA concentrations on S. ellipsoideus *BAW_2 and* S. pastorianus, *Y-IV with different CW concentrations in NG medium. 1% BAW_2 + 10% CW (–•–) 25% CW (–▲–) 50% CW (–■–) Y-IV + 10% (–O–) 25% CW (–△–), 50% (–□–) after 24 h.*

Plate 1

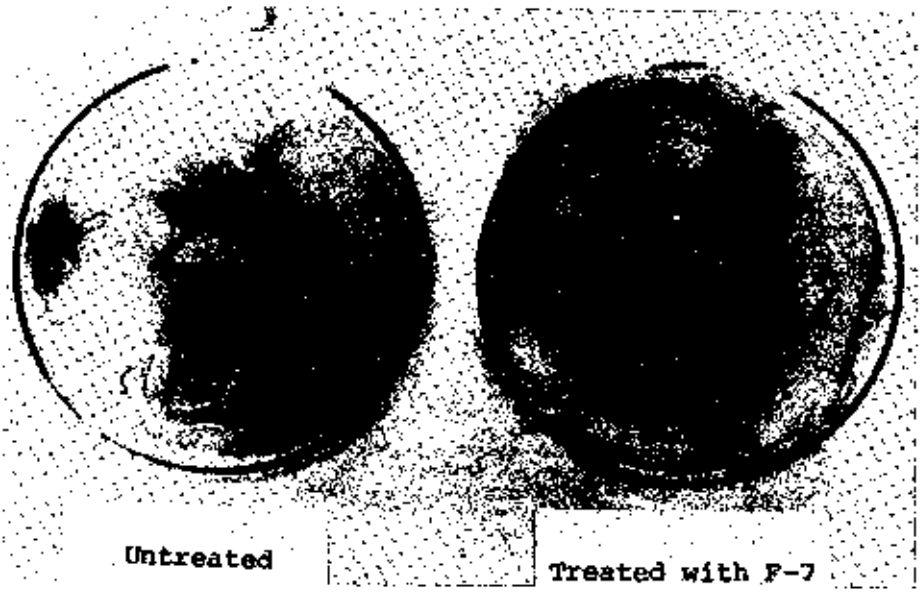

Fig. 1. Effect of cell filtrate of F-7 on the corn host plant after 7 days.

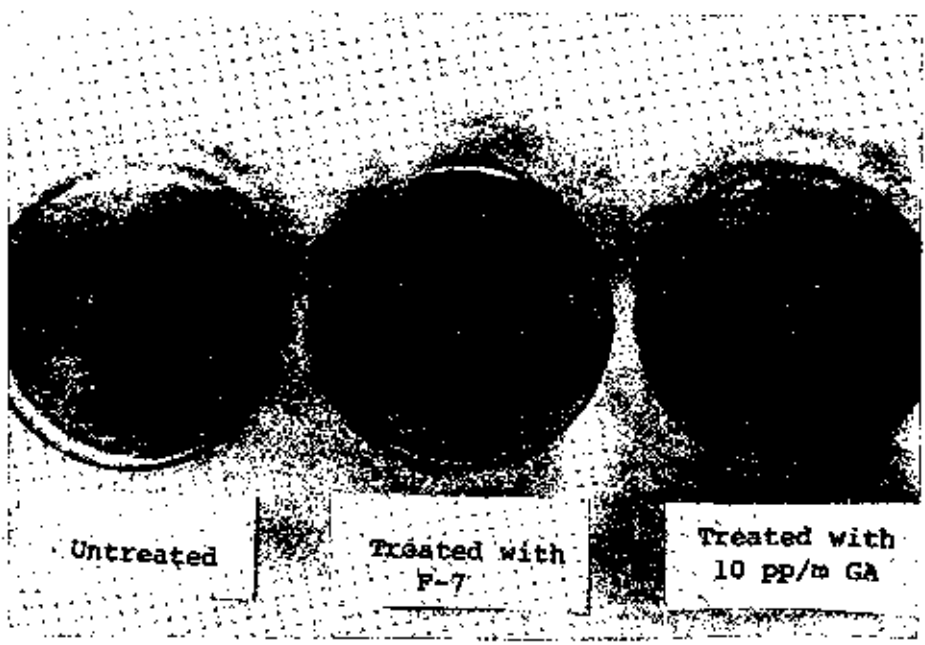

Fig. 2. Effect of F-7 and gibberellic acid on the corn host plant after 7 days.

Plate 2

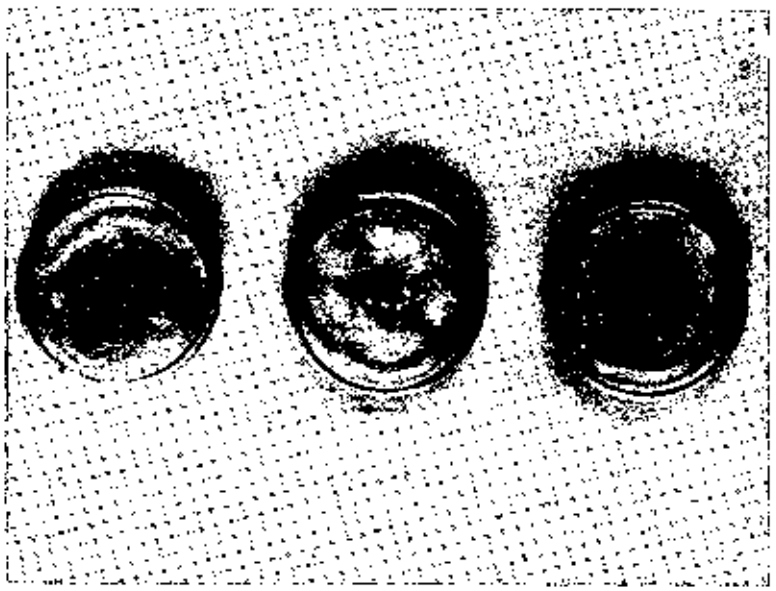

Fig. 3. Mycelia of F-7 on Czapek's Dox Medium after 14 days.

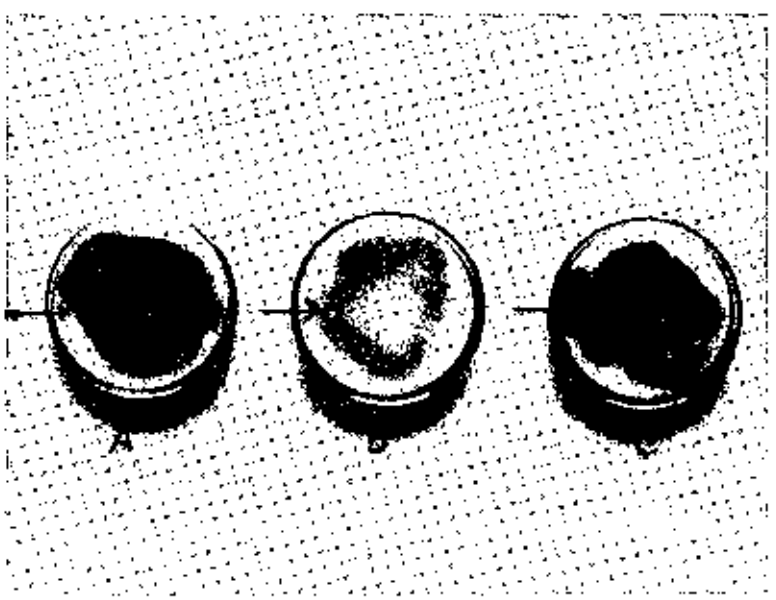

Fig. 4. Dark zones of F-7 on Czapek's Dox Agar with 3% $CaCO_3$ after A-14 days; B-10 days, C-13 days.

Plate 3

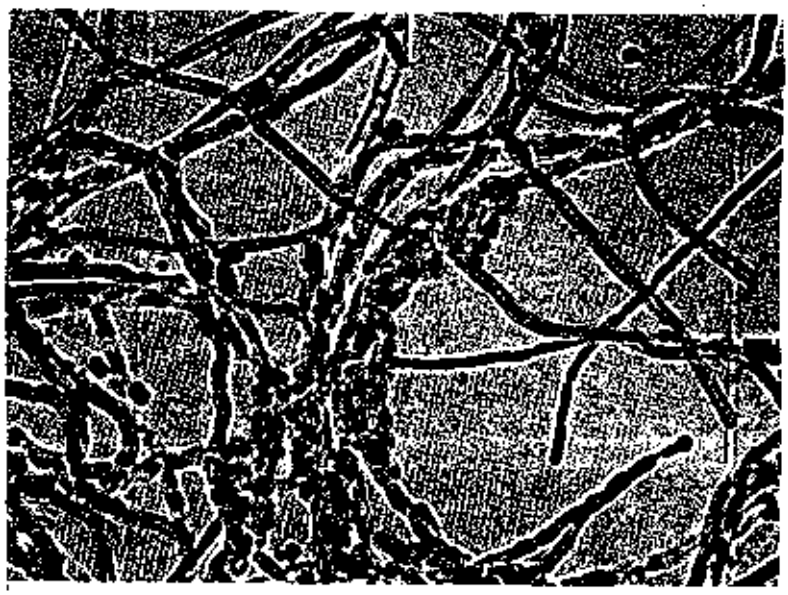

Fig. 5. *Aerial mycelia from a 7-day-old culture of F-7 with microconidia in chains. (Approx. x 400).*

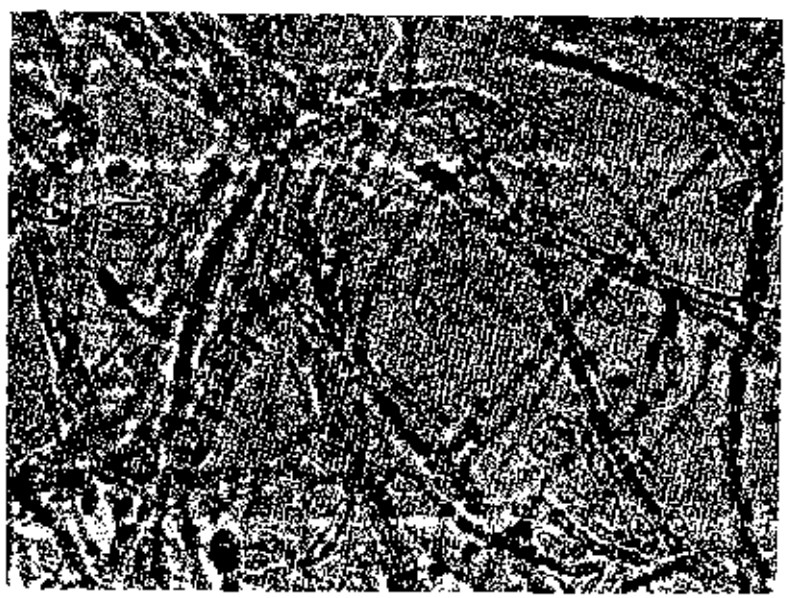

Fig. 6. *Mycelia from a 14-day-old culture of F-7 with microconidia at the tips and sides of hyphae and few macroconidia (Approx. x 400).*

Plate 4

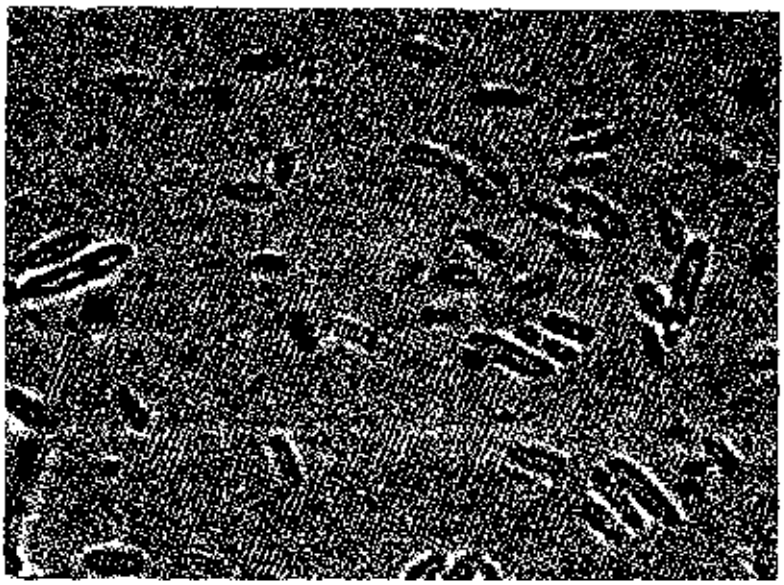

Fig. 7. Macroconidia from a 4-week-old culture of F-7 at 37° C.

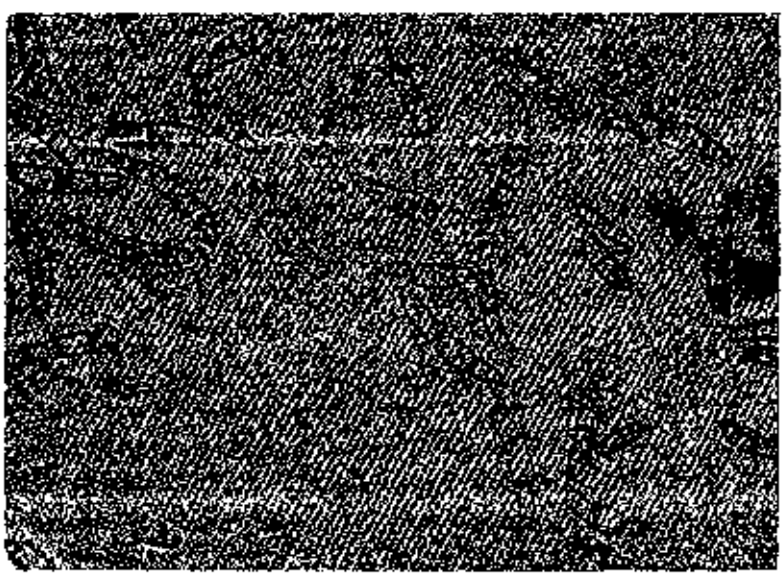

Fig. 8. Macroconidial propagation from a 4-week old culture of F-7 at 27° C.

TWO-STAGE PROCESS OF ETHANOL PRODUCTION FROM SWEET POTATO FLOUR AND RICE BRAN USING *ASPERGILLUS AWAMORI* AND IMMOBILIZED YEAST

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ABSTRACT

A two-stage process was developed for producing ethanol from sweet potato flour and rice bran. The first stage consisted of starch saccharification by the amylolytic mold *Aspergillus awamori* NRRL 3112. The latter was used to saccharify powdered sweet potato tuber (15% w/v) and rice bran (6% w/v) after thinning with bacterial alpha-amylase using a 3.5-liter airlift fermenter. Saccharification efficiencies of 90.13-90.20% were observed after 12-48 hours of batch fermentation under the following optimized conditions: pH 5.5, 21% (w/v) solids level and 10% (v/v) inoculum size. After 48 hours of batch culture, 90% of the starch in the potato flour substrate was converted into sugars at pH 5.5 and 30°C. Glucose was the major constituent of the hydrolysate.

Continuous fermentation of the centrifugal hydrolysate in the second stage with a local yeast isolate immobilized on "ipil-ipil" *(Leucaena leucocephala)* sawdust and shavings at 42°C, at a dilution rate of $0.13h^{-1}$ resulted in an alcohol content of 5.2% w/v, with a fermentation efficiency of 79.5%. A maximum ethanol level of 5.6% (w/v) was obtained in the 'beer' after 19 hours of fermentation with a cell loading of 4×10^{10} cells per gram of immobilizing material corresponding to a fermentation efficiency of 85.0%. Shorter fermentation times resulted in lower ethanol yields.

INTRODUCTION

The enzymatic conversion of starch into sugar utilizes alpha-amylase (1,4—α-D-glucan glucanohydrolase, EC 3.2.1.1) for starch thinning and glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) for hydrolyzing thinned starch into glucose. The latter sugar may then be fermented into ethanol by means of the conventional method employing yeast (Kosaric *et al.*, 1980; del Rosario, 1983). However, this enzymatic process requires that the two amylolytic enzymes be separately produced.

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An alternative process, which is sometimes called the 'Amylo' process, does not employ added enzymes but uses enzymes produced *in situ* by an amylolytic microorganism. Sreekantiah and Satyanarayana Rao (1980) had studied the conversion of starch from potato or cassava into ethanol using a mixed culture of *Rhizopus niveus* and yeast. This mixed-culture process is potentially simpler and more economical than the enzymatic process because the need for separately producing the amylolytic enzymes is obviated. However, the amylolytic and alcohol-producing microorganisms need not be combined in a fermentor for a single-stage process but can be placed in two separate fermenters. Such a sequential or two-stage process, which allows the first-stage microbial saccharification and second-stage alcohol fermentation to be separately optimized, has recently been employed by del Rosario and Wong (1984) for converting dextrinized cassava starch into ethanol.

The present paper deals with the two-stage microbial conversion of sweet potato (*Ipomoea batatas* (L.) Lam) and rice bran into ethanol using the 'Amylo' process. The optimum pH, subtrate solids level and inoculum size were determined for the first-stage saccharrification of a mixture of sweet potato flour and rice bran by the amylolytic mold *Aspergillus awamori*. The second-stage fermentation of the hydrolysate into ethanol was optimized in terms of dilution rate and yeast cell concentration. The extent of starch hydrolysis in the first stage was determined by sugar analysis of the hydrolysate using high pressure liquid chromatrography (HPLC).

MATERIALS AND METHODS

Organisms and Substrate Preparation

Aspergillus awamori NRRL 3112 was kindly given by the Northern Regional Research Center, U.S. Dept. of Agriculture, Peoria, Illinois, U.S.A. A local strain of *Saccharomyces* sp. (BB 1945), which was isolated from a commercial sample of sugarcane molasses, was used for alcohol fermentation (Bugarin, 1983).

Fresh sweet potatoes were sliced to a thickness of 5 mm, dried at 105°C for 24 hours and ground in a Wiley Mill. The resulting flour was sieved (100 mesh) prior to enzymatic treatment. Bacterial amylase Novo 240 Liquid 'Ban 240L', an alpha-amylase preparation from Novo Enzyme Corp. (Bagsvaerd, Denmark) with an enzyme activity of $(8 \pm 1) \times 10^5$ DP/mL, was used for starch thinning.

Starch Saccharification

For the batch saccharification of a mixture of Sweet Potato Powder (SPP) and rice bran a 3.5 mL airlift fermenter was used. SPP and rice bran (3.5:1 ratio) were suspended in water, the amount of which was three times the combined weights of the SPP and rice bran. The fermentation medium contained the following: SPP, 15%; rice bran, 6%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.15%; $MgSO_4$, 0.025%. The pH was adjusted to 6.5 and Novo alpha-amylase, which was described above, was added at a dosing rate of 0.002 and enzyme solution per gram of solid.

The suspension was heated at 80°C with continuous stirring for 30 min. After cooling, the pH was adjusted to 5.5 and the suspension was sterilized for 15 min at 15 psig. The suspension was cooled and transferred into the airlift fermenter aseptically using a peristaltic pump and the inoculum of *A. awamori* NRRL 3112 was added. Air was supplied to the fermentation liquid at the rate of one volume of air per volume of liquid per min (1 vvm) as measured by a flowmeter. The pH of the medium was automatically controlled using a New Brunswick pH controller. Sampling of the fermenting suspension was done at regular intervals for 96 h.

The following parameters were tried for optimization of the saccharification process: Three pH levels, namely: pH 5.0, 5.5 and 6.0; 3 inoculum levels, namely: 10%, 15% and 20% (v/v); and 2 substrate solid levels, namely: 21% and 26% w/v.

Yeast Immobilization

A 30-mL suspension of yeast cells was prepared by adding sterile distilled water to 2-day old MYGP agar yeast slants (5 mL/slant) and scraping the yeast with an inoculating loop. The yeast suspension was added to a 1-liter Erlenmeyer flask containing 300 mL sterilized molasses medium (5% w/v total sugars). Sterilized supports were added in weighed amounts (to be discussed later). A control flask containing the microorganism but without the support material was also run. The flask with suspension was incubated for 24 hours in the shaker (~240 rpm) for yeast immobilization. After 24 hours the suspension with the added support was allowed to stand for 30 minutes in order to separate the support containing the absorbed yeast from the supernatant of free cells. The latter was decanted into another flask and the cell count was taken. The cell count of the control was also determined.

Microscopic examination of the yeast immobilization process was done using an Olympus microscope Model BHC. The latter, in combination with a haemocytometer, was used for determining yeast cell counts.

Alcoholic Fermentation

The saccharified mixture of sweet potato flour and rice bran was centrifuged in a basket centrifuge. The pH of the solids-free hydrolysate was adjusted to 4.5. Continuous fermentation was done using yeast (local isolate BB1945) immobilized by adsorption on "ipil-ipil" wood (*Leucaena leucocephala*) sawdust and shavings. A 700-mL glass column (4.5 cm dia. x 47 cm high) was used to hold the immobilized yeast during the alcoholic fermentation (Fig. 1). Two initial yeast cell concentrations were used, namely: 8.3×10^8 cells/mL and 1.3×10^9 cells/mL and three dilution rates were tried, namely: $0.13h^{-1}$, $0.18h^{-1}$ and $0.22h^{-1}$.

Analytical Procedures

Moisture Determination

The moisture content of sweet potato flour and rice bran was determined using standard methods of analysis (AOAC, 1970).

Starch Content

Starch was analyzed using the method of Sin-I (1974).

Alcohol Determination

The alcohol content was determined by gas chromatography using a Shimadzu gas chromatograph (AGPRIF model). Absolute ethyl alcohol (Merck) was used as standard.

Sugar Analysis

Reducing sugars were determined by the method of Miller (1959) using glucose as standard.

High Pressure Liquid Chromatography (HPLC) was used for the carbohydrate analysis of hydrolysates. The starch hydrolysates were diluted and pre-filtered using ordinary Whatman filter paper. Final filtration was done using a PM 30 Amicon ultrafiltration membrane. Doubly-glass-distilled water was used as solvent. It was allowed to pass a Solvent Clarification Kit for separation of particulates. The solvent was degassed before feeding into the solvent supply line. The filtered samples were injected into a Waters Liquid Chromatography system which consists of 6000 A pump system, universal injector system, differential refractometer-RI 401 and Sugar-PAK 1 column. The entire unit was preheated for 15-20 minutes. Analysis was carried at 90°C with a chart speed of 0.50 cm/min and a flowrate of 0.50 mL/min. Attenuation was set at 8x.

Details of the material preparation methods, fermentation procedures and analytical techniques used in this study are similar to those described by Bugarin (1983).

A schematic drawing of the experimental set-up for converting sweet potato starch into ethanol is given in Figure 1.

RESULTS AND DISCUSSION

Starch Saccharification

Based on chemical analysis, the moisture content of the sweet potato flour used in the present study was 2.54% and the starch content was 75.5%-85.4% on dry basis. Rice bran had 1.62% moisture and 17.3%-26.0% starch (dry basis).

Results of the first-stage saccharification of the sweet potato flour-rice bran mixture using *Aspergillus awamori* NRRL 3112 are shown in Fig. 2 in terms of the concentration of reducing sugar (R.S.) as influenced by variation in pH using 21% (w/v) solids level and 10% (v/v) inoculum size. The maximum concentration of R.S. obtained was 14.4% with a corresponding saccharification efficiency of 90.2% after 42 hours of microbial action at pH 5.5. Osorio (1981); Nithiandam *et al* (1981), and Acabal (1983) reported similar findings. Nithiandam *et al* (1981) have reported that the saccharifying enzyme glucoamylase has a maximum stability at pH 5.5 and Osorio (1981) reported

similar pH optimum. Acabal (1983) obtained highest volumetric activities of the saccharifying enzyme at pH 5.5

Reducing sugar concentrations as influenced by variation in inoculum size are shown in Fig. 3. The maximum value obtained was 13.7% (w/w) with a saccharification efficiency (S.E.) of 85.6% after 45 hours fermentation time at 10% (v/v) inoculum size. Meyrath (1962), has done experiments to explain the observation that the growth rate and maximum yield of mycelium of *Aspergillus oryzae* of small inocula increased over those observed with culture inoculated with much larger inocula. It was observed that growth inhibiting substances were excreted by *Aspergillus oryzae* cultures. During the study of saccharifying enzyme production, Acabal (1983) observed depletion of reducing sugars at 20% v/v inoculum size. Throughout the run, the levels of reducing sugars were lower as compared to the concentrations obtained at 10% inoculum size. In addition, volumetric activities of glucoamylase and protein contents were relatively lower at 20% inoculum level. These results suggest that inoculum levels higher than 10% at 21% solids level and pH 5.5 are not favorable for the saccharification stage.

Values of the reducing sugar (R.S.) concentration as influenced by variation in substrate solids level are shown in Fig. 4. Higher R.S. values were obtained at 26% level but higher saccharification efficiencies (S.E.) were obtained at 21% level. An S.E. value of 88.4% was obtained after 36 hours at 21% solids level. On the other hand, at 26% level, a lower S.E. value of 73.5%, was obtained. Studies involving measurement of activities of the saccharifying enzyme were conducted by Smiley (1974), Osorio (1981) and Acabal (1983). Using the same strain of mold, they reported high activities of the enzyme at 20% substrate solids level.

Yeast Immobilization

As shown in Table 1, at two initial yeast cell concentrations, namely 8.3×10^8 and 13.0×10^8, the extent of immobilization was 57.6 and 48.7%, respectively with the corresponding yeast loading of 6.2×10^9 and 9.2×10^9 cells/g support, respectively. The percentage of yeast immobilized is higher (57.6%) for a less concentrated system (lower initial cell count) as compared with a more concentrated system which has 48.7% immobilization.

Microscopic observations showed that during immobilization the yeast cells were physically trapped inside the pores of the wood particles. The use of a very concentrated suspension of yeast, i.e., high initial count, did not enhance immobilization; a certain amount of support could only take up a maximum number of cells.

Ethanol Fermentation

Results of the second-stage ethanol fermentation using the immobilized yeast are presented in Fig. 5 in terms of the alcohol concentration in the fermented product 'beer' at two different cell loadings. A maximum alcohol concentration of 5.21% (w/v) and a fermentation efficiency (F.E.) of 79% were obtained at a cell loading of 6.2×10^9 cells/g. However, lower values of alcohol concentration (4.5% w/v) and F.E. (69.2%) were observed at a higher cell loading of 9.2×10^9 cells/g.

Alcohol levels obtained at varying dilution rate are shown in Fig. 6. A maximum alcohol concentration of 4.60% w/v and a corresponding fermentation efficiency of 70.9% were observed at 8 ... fermentation time at a dilution rate of $0.13h^{-1}$. Results of these experiments are comparable to those of Bland *et al.* (1982), Williams and Munnecke (1981) and Ghose and Tyagi (1979). These workers observed a decreased ethanol concentration with increasing dilution rate. This implies that at high dilution rate (flow rate divided by fermenter volume), the substrate passed out of the system incompletely reacted. Due to the short hydraulic residence time the yeast was not able to completely ferment the sugars into alcohol.

Results for the unsterilized and sterilized conditions are shown in Fig. 7. A higher alcohol level of 5.6% (F.E. 85.0%) was observed under sterilized conditions. A lower value, 5.2% (F.E. 79.5%) was observed under unsterilized conditions. Microscopic examination showed a high population of cocci and occasional rods in the 'beer' under unsterilized conditions. Unfortunately, the 0.4% difference in alcohol level is not substantial in view of the large experimental errors encountered. More definitive experiments should be performed in order to confirm the results.

Analysis of Starch Hydrolysate by HPLC

High pressure liquid chromatography (HPLC) was used for analyzing the starch hydrolysate obtained in the first-stage airlift fermenter. The carbohydrate components of the hydrolysate were identified by the "spiking" technique. A typical high pressure liquid chromatogram is presented in Fig. 8 and shows seven peaks together with the internal standard (sorbitol). Peaks 5, 6 and 7 were identified as maltose, glucose and fructose, respectively. Peak 1 is probably dextrin while peaks 2, 3 and 4 are oligosaccharides. spiking with maltopentaose, maltotetraose and maltotriose enhanced peaks 2, 3 and 4, respectively. However, identification of these peaks is not conclusive.

Some of the results of the sugar analysis of the starch hydrolysate are given in Table 2. The latter shows that both the glucose concentration and mass ratio of glucose to total sugars increased with fermentation time. This can be attributed to the cumulative effects of starch saccharification brought about by amylolytic enzymes secreted in the medium by *Aspergillus awamori*. The initial medium or suspension of sweet potato flour and rice bran (zero time) contained 20.3 mg/mL (or 2.03% w/v) glucose and a mass ratio of glucose to total sugars equal to 0.70. As fermentation proceeded, the glucose concentration increased as a result of the enzymatic hydrolysis of starch dextrins and oligosaccharides into glucose.

SUMMARY AND CONCLUSIONS

Studies were conducted on the two-stage production of ethanol from sweet potato flour (15% w/v) and rice bran (6% w/v) after thinning with bacterial alpha-amylase. The first stage consisted of saccharification of the starch content of the mixed substrates in a 3.5-liter airlift fermenter using the amylolytic mold *Aspergillus awamori* NRRL 3112. The optimum pH, substrate level and inoculum size were determined. The highest saccha-

rification efficiencies of 90.13-90.20% were observed after 12-48 hours of batch fermentation at the optimized conditions of pH 5.5, 21% (w/v) solids level and 10% (v/v) inoculum size.

Mass balance determination showed that at 21% and 26% substrate levels, the solid residue obtained was 35.8% and 37.3%, respectively, of the dry weight of the substrate. The residue consisted of fungal mycelium, crude fiber, ash and unhydrolyzed starch. Ninety percent of the starch in the sweet potato flour was hydrolyzed into sugars after the first-stage saccharification at pH 5.5 and 30°C. Glucose was the major constituent of the centrifuged (solids-free) hydrolysate.

The centrifuged hydrolysate, after nutrient addition and pH adjustment to 4.5, was passed through the second-stage continuous-flow ethanol fermenter, which was maintained at 42°C. The fermenter was a 700-mL glass column containing a local yeast isolate (BB 1945) which had been immobilized on 'ipil-ipil' (*Leucaena leucocephala*) sawdust and shavings. Yeast cell immobilization was studied at two different initial cell concentrations and expressed in terms of extent of immobilization and yeast loading. Three dilution rates were tried for alcohol fermentation, namely 0.13, 0.18 and 0.22h^{-1}.

At a dilution rate of 0.13 h^{-1} an alcohol content of 5.2% w/v was obtained in the 'beer' corresponding to a fermentation efficiency of 79.5%. A maximum ethanol level of 5.6% (w/v) was obtained after 19 hours (residence time) with a cell loading of 4 x 10^{10} cells per gram of immobilizing material. This corresponds to 85.0% fermentation efficiency.

The two-stage process, although promising, needs further optimization and scale-up studies in order to assess its long-term potential for large-scale applications.

ACKNOWLEDGMENTS

The authors are grateful for a scholarship grant from the Philippine Council for Agriculture and Resources and Development (PCARRD) and additional thesis support from the Philippine Development Scholarship Program (PDSP) of the University of Life given to R. Bugarin. The kind technical assistance for chemical analysis provided by Prof. L.S.P. Madamba, Mmes. E. Flavier, R. So and V. Papa-Migo is also acknowledged.

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Table 1. Immobilization data at two different yeast cell concentrations in the second stage of a two-stage fermentation.

Initial Cell Count $x10^{-8}$	Total Number of Cells Immobilized $x\ 10^{-8}$	Percent Immobilization	Cell Loading per gram Support $x\ 10^{-9}$
13.0	6.9	48.7	9.2
8.3	5.3	57.7	6.2

Table 2. Sugar analysis of the starch hydrolysate using high pressure liquid chromatography.

Fermentation time, hours	Concentration mg/mL Glucose	Maltose	Fructose	Mass ratio of glucose to total sugar
0	20.3	6.5	2.0	0.70
pH 5.0				
12	35.2	–	1.6	0.96
34	41.3	2.0	–	0.95
pH 6.0				
18	37.8	–	1.9	0.95
20	43.1	–	0.8	0.98
24	50.0	–	0.8	0.98
37	52.3	–	5.1	0.91

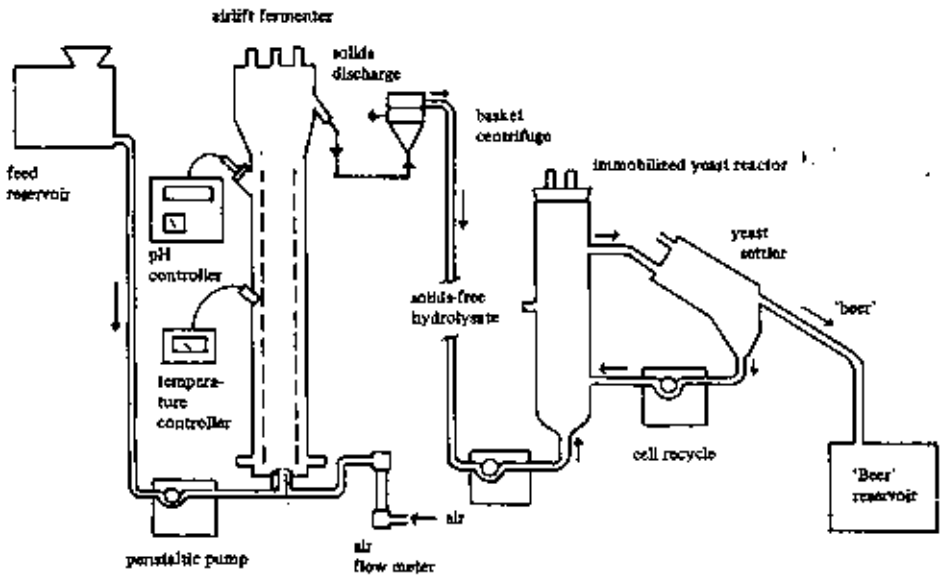

Fig. 1. Two-stage process for ethanol production from alpha-amylase treated sweet potato root flour.

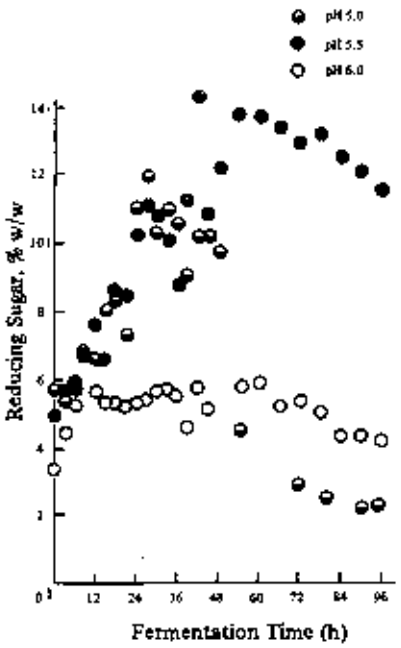

Fig. 2. Concentration of reducing sugars in the culture medium during saccharification of sweet potato flour by A. awamori *RNNL 3112 as influenced by variation in pH using 21% solids level of substrate and 10% (v/v) inoculum size.*

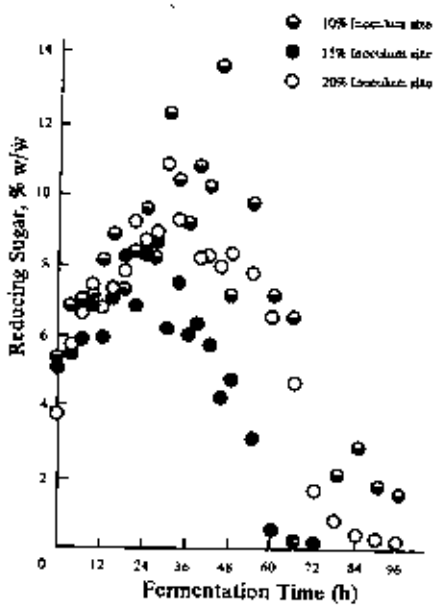

Fig. 3. Concentration of reducing sugars on the culture medium during saccharification of sweet potato flour by A. awamori *NRRL 3112 at pH 5.5 as influenced by variation in inoculum size using 21% (w/w) solids level of substrate.*

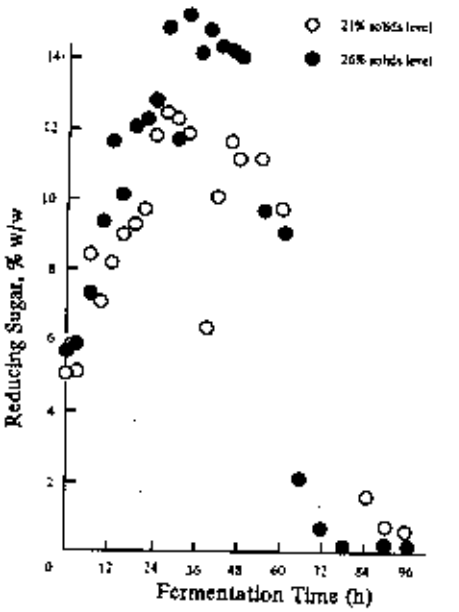

Fig. 4. Concentration of reducing sugars on the culture medium during saccharification of sweet potato flour by A. awamori *as influenced by variation in solids levels of substrate using 10% (v/v) inoculum size at pH 5.5.*

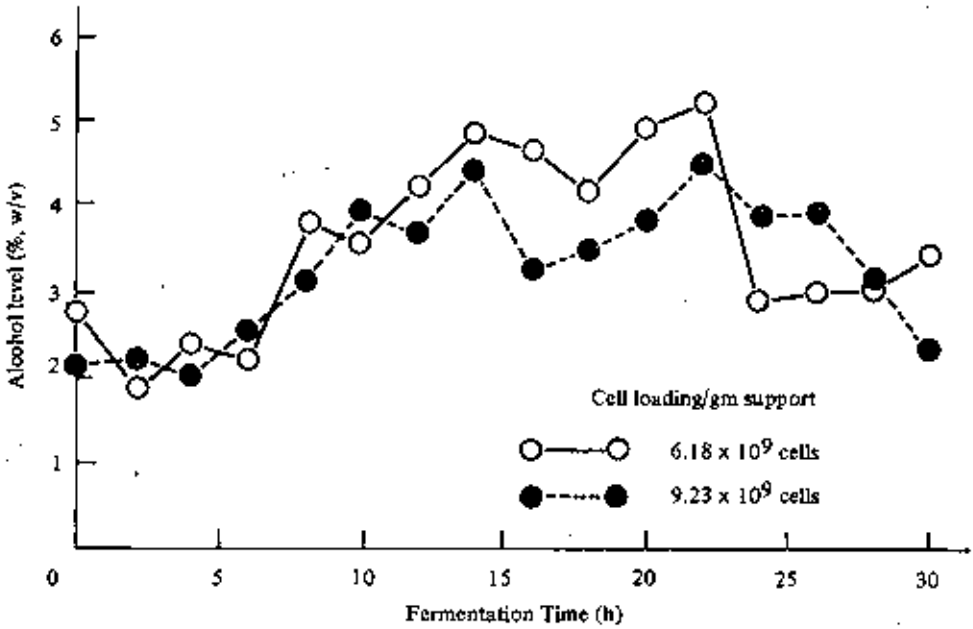

Fig. 5. Ethanol concentrations at different cell loadings in the second stage of the two-stage fermentation of sweet potato flour under unsterilized conditions.

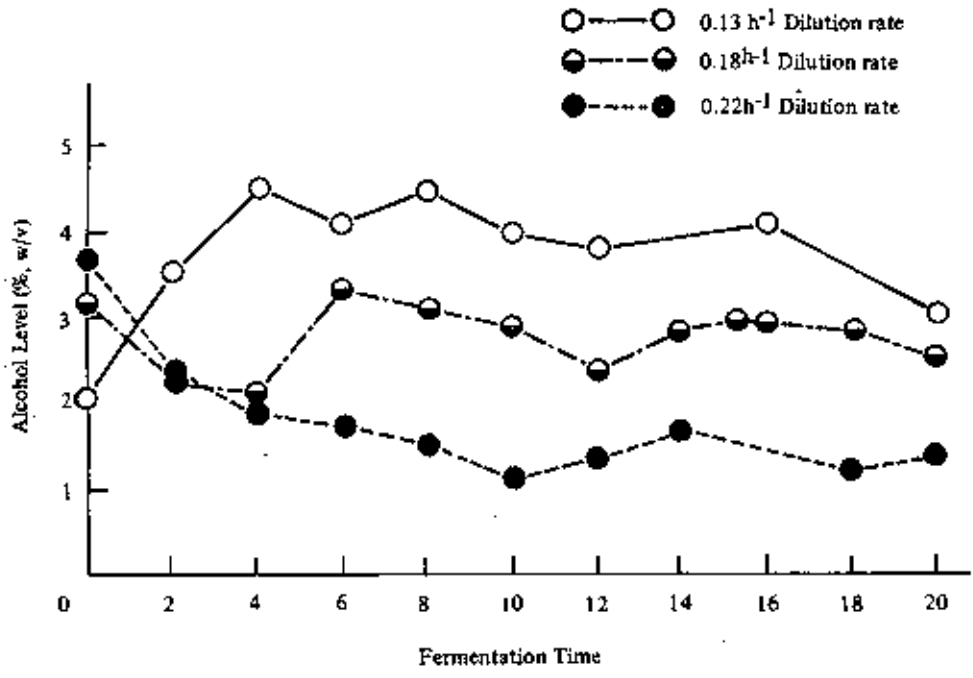

Fig. 6. Levels of ethanol of varying dilution rates in the second stage of the two-stage fermentation of sweet potato flour under unsterilized conditions.

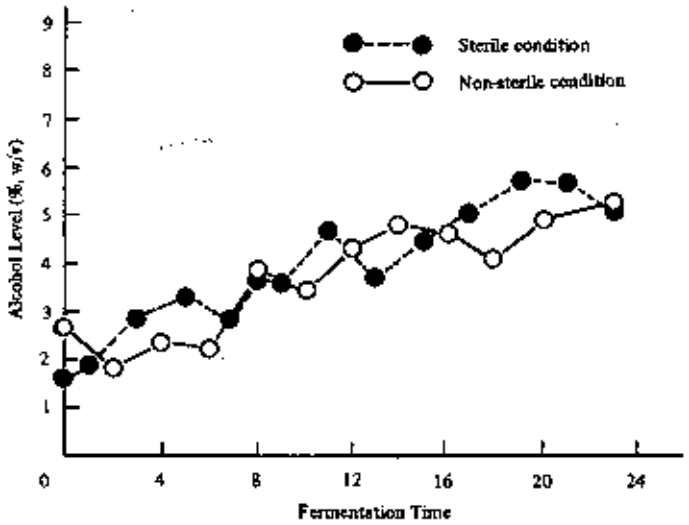

Fig. 7. Ethanol concentration under sterilized and unsterilized conditions in the second stage of the two-stage fermentation of sweet potato.

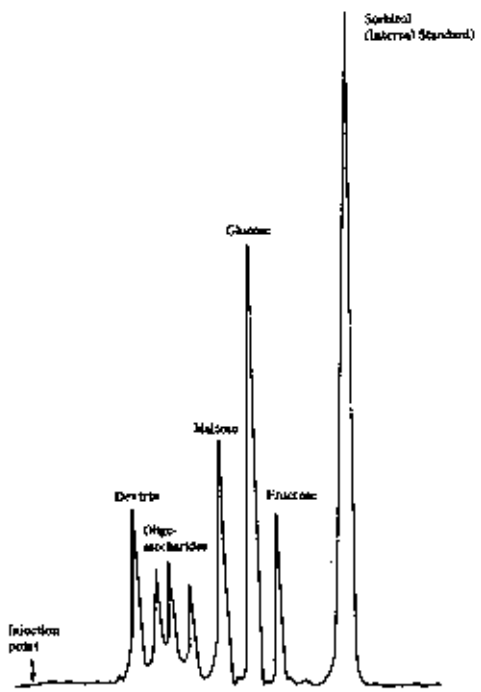

Fig. 8 HPL Chromatogram of 10 mL hydrolysate plus 10 mL sorbitol (5 mg/mL) at a flowrate of 0.5 mL/min through a sugar Pak I at 90°C.

THE ISOLATION AND IDENTIFICATION OF HOLOTHURINS A AND B OF THE PHILIPPINE SEA CUCUMBER, *HOLOTHURIA PULLA* SELENKA

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ABSTRACT

High performance liquid chromatography with Silica gel ODS-Q3, Toyo Pearl HW40, Develosil 30/50, and Develosil C8 15/30 columns and either 60% methanol or 30% acetonitrile in mobile phase isolated two saponins from the crude extracts of the body wall of the Philippine sea cucumber, *Holothuria pulla* Selenka. Thin layer chromatography and spectroscopic analyses demonstrated the saponins to markedly resemble Holothurins A and B. The HPLC-derived holothurins occurred in 1:6 ratio with the major Holothurin B readily crystallized in butanol, holothurin A in methanol. A most probable third saponin with the same Rf value as that of Holothurin A was indicated on TLC.

INTRODUCTION

Crude holothurin from the body walls of the Philippine sea cucumber, *Holothuria pulla* Selenka had been derived as alcohol extracts and demonstrated to possess significant hemolytic activity and mutagenicity and clastogenicity potentials (Pocsidio, 1983, 1983a and 1986). In this study, the crude holothurin was purified by high performance liquid chromatography. The saponins yielded by the procedure were identified by their hemolytic indices, thin layer chromatograms, reaction in the potassium rhodizonate test, melting points, and spectroscopic activities.

MATERIALS AND METHODS

Crude holothurin (10g) was partitioned into ethylacetate-H_2O mixture (1:1) and the water phase afterwards thrice partitioned into n-BuOH-H_2O mixture (1:1). The n-BuOH phase was separated and the solvent evaporated under reduced pressure. The residue was dissolved in 60% methanol and passed through a silica gel ODS-Q3 column (4.5 x 20 cm) using the same solvent to elute the saponins. Separation of Holothurins A and B was accomplished in a column of Toyo Pearl HW40 (Toyosoda, 2.8 x 40 cm). Colored contaminants were removed in Develosil ODS 30/50 column (Nomura Kagaku, 0.6 x 25 cm) and further purification was done in Develosil C8 15/30 column (Nomura Kagaku, 2.8 x 40 cm). HPLC in the three columns was with 30% acetonitrile.

Detection of the saponin was in Merck Silica Gel 60 precoated plates developed in $CHCl_3$-MeOH-H_2O (6:4:1) and sprayed by H_2SO_4 vapor against reference Holothurins A and B from Dr. Kitagawa of the Osaka University, Japan. 1% mouse blood was also sprayed directly on the TLC plates for saponin detection. The potassium rhodizonate test (Schneider and Lewbart, 1956) detected for the presence of sulfate. Calculation of

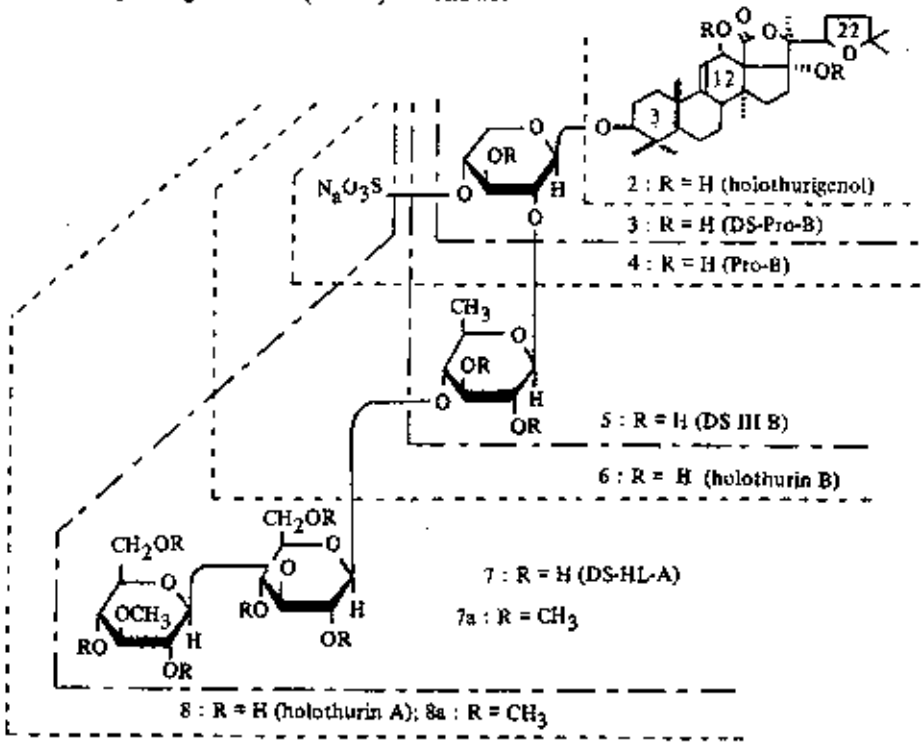
the hemolytic index 2% mouse RBC/gram dry crude holothurin against Merck Standard Saponin used the formula suggested by Fujita and Nishimoto (1952).

Holothurin A was dissolved in warm methanol and crystals were allowed to form upon cooling. Holothurin B was dissolved in water-saturated butanol. Absolute butanol was added until crystals started to form. In room temperature conditions, crystals of Holothurin B were allowed to grow.

Melting points of the crystals were determined on the Yanaco Micromelting Point apparatus. Spectroscopic data were obtained as follows: UV in MeOH, IR in KBr, specific rotation of D-holothurin in MeOH, ^{13}C-NMR and PMR in d5-pyridine. The various spectroscopic measurements were done of both holothurins except for the PMR of Holothurin A.

RESULTS AND DISCUSSION

Holothurin B, $C_{41}H_{63}NaO_{17}S$, is 3–0–(2′–0–β–D–quinovopyranosyl-β–D-xylopyranosyl) – holothurigenol 4′–O–sodium sulfate and Holothurin A, $C_{54}H_{85}NaO_{27}S$, 3–0–[β–D–3–0–methylglucopyranosyl– (1 → 3)– β – D–glucopyranosyl) (1 → 4) – β – D–quinovopyranosyl (1 → 2)– β –D–xylopyranosyl] – holothurigenol 4′–0–sodium sulfate (Kitagawa *et al*, 1981 and 1981a). Holothurin A corresponds to the 4" –0– [3" '–0– (β–D–3" "–0–methyl-glucopyranosyl)–β – D–glucopyranoside] of Holothurin B. The structural formulas of the triterpene oligoglycosides and their different moeities were drawn by Kitagawa *et al.* (1981a) as follows:



In the present study, ten gram sample of crude holothurin yielded approximately 125 mg Holothurin B and 20 mg Holothurin A. Runs through the Toyo Pearl and Develosil columns eluted Holothurin A ahead of Holothurin B. The spectral and other results agree closely with those of previous works (Yasumoto *et al,* 1967; Kitagawa *et al* 1978, 1981, and 1981a). The very slight differences between the data obtained in this study and those by Kitagawa *et al* may probably be due to the small amounts of Holothurin A used for the measurements. Because of the size of the molecule, Holothurin A was not subjected to PMR analysis. However, the data provided enough evidence as to the identity of the compounds.

Holothurin B: Colorless needles: Hemolytic index mouse RBC/gram dry crude holothurin, 111,697: Rf, 0.68; potassium rhodizonate test, positive; melting points, 221-223 °C; UV (MeOH), transparent above 210 nm. Specific rotation of D-Holothurin B (MeOH), −14.5°; IR (KBr), 3400, 1740, 1630, 1230, 1065, and 830 cm^{-1} (Fig. 1); ^{13}C-NMR (Table 1) ·PMR (Fig. 3). All the signals elicited from DS-Pro-B, a moeity of Holothurin B according to Kitagawa *et al* (1978) were shown in the PMR.

Holothurin A: Colorless needles; Hemolytic index mouse RBC/gram dry crude holothurin, 193,962. Rf, 0.54; potassium rhodizonate test, positive; melting point, 240-242°C; UV (MeOH), transparent above 210 nm; Specific rotation of D-Holothurin A (MeOH), − 10.0°; IR (KBr), 3420, 1750, 1625, 1220, and 1065 cm^{-1} (Fig. 2); ^{13}C-NMR (Table 1).

Elyakov *et al* (1973) reported the occurrence of Holothurins A and B in *Holothuria pulla* as identified on TLC. In the present study, HPLC and TLC not only yielded the two holothurins for spectroscopic analyses but also a most probable third saponin, the minimal amounts of which, however, did not permit its identification. It appeared on TLC plates with the same Rf value as that of Holothurin A although it was eluted in the later fractions.

A significance of this study is that it introduces a new method for the purification of holothurin. It is also the first report on crystals of Holothurins A and B of *H. pulla.*

The purification procedure is fundamental to future research especially on the medical importance of holothurin. Earlier works had demonstrated effectivity of crude holothurin against some tumors, tumor and cancer cells (Nigrelli and Zahl, 1952; Sullivan *et al,* 1955; Cairns and Olmsted, 1973; Colon *et al,* 1974; Kuznetsova *et al,* 1982) as well as against fungi (Shimada, 1960).

SUMMARY AND CONCLUSIONS

1. Crude holothurin of the Philippine sea cucumber, *Holothuria pulla* Selenka solvent partitioned in ethylacetate-H_2O and in n-BuOH-H_2O mixtures and later sequentially passed through HPLC columns yielded pure holothurins which readily crystallized in either butanol or methanol.

2. Spectral analyses, the determination of hemolytic indices and melting points, thin layer chromatography, and the potassium rhodizonate test conclusively provided evidences for the identification of Holothurins A and B.

ACKNOWLEDGEMENTS

The author is grateful to Dr. Takeshi Yasumoto of the Tohoku University for his invaluable advice, Dr. Isao Kitagawa for gifts of reference saponins and Mr. Michio Murata for obtaining the spectroscopic data. The study was conducted on a Matsumae International Foundation Research Fellowship in the Tohoku University, Sendai, Japan.

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Table 1. ^{13}C-NMR data of holothurins A and B of *H. pulla*.

Carbon	Holothurin B	Holothurin A
1	35.745	35.732
2	27.437	27.441
3	88.690	88.748
4	40.074	40.462
5	52.711	52.665
6	20.358	20.318
7	28.139	28.142
8	40.893	40.929
9	153.803	153.500
10	39.723	39.644
11	115.543	115.197
12	71.490	71.874
13	58.795	58.737
14	45.924	27.150
15	27.100	27.150
16	38.494	38.418
17	89.743	89.624
18	174.455	174.220
19	18.837	18.917
20	86.642	86.529
21	22.523	22.537
22	80.675	80.574
23	36.432	36.433
24	28.666	28.668
25	81.377	81.274
26	28.666	28.668
27	28.139	28.142
28	21.236	21.486
29	27.437	27.441
30	16.731	16.698
1'	105.773	105.680
2'	83.425	83.260
3'	76.697	76.312
4'	75.176	74.910
5'	64.119	64.525
1''	105.188	105.330
2''	75.819	75.728
3''	77.457	76.720
4''	76.697	86.529
5''	73.421	71.874
6''	18.545	17.224
1'''		105.155
2'''		73.859
3'''		88.339
4'''		70.473
5'''		77.479
6'''		62.065
1''''		104.513

Table 1. Continued . . .

Carbon	Holothurin B	Holothurin A
2'''		74.910
3'''		87.814
4'''		69.364
5'''		78.122
6'''		61.656
3''' OMe		60.722

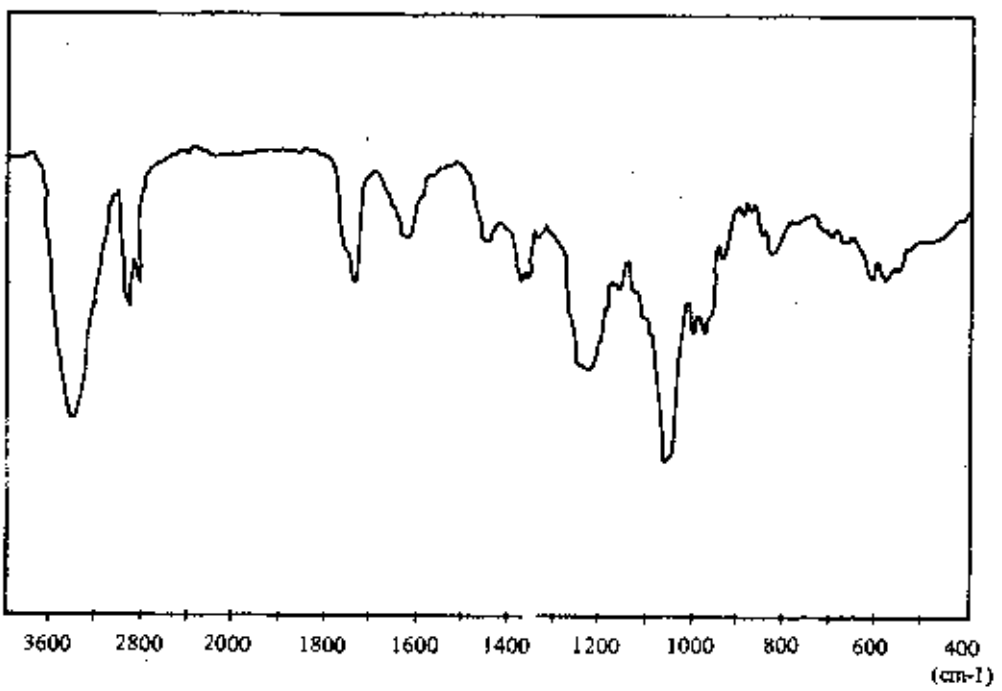

Fig. 1. IR spectrum of Holothurin B of H. pulla.

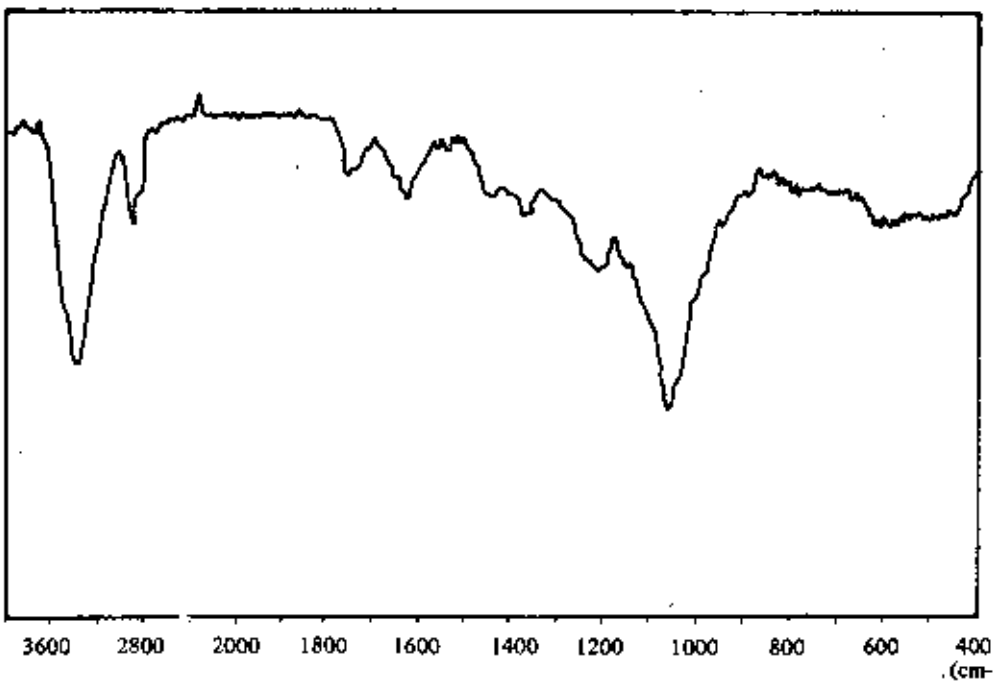

Fig. 2 IR spectrum of Holothurin A of H. pulla.

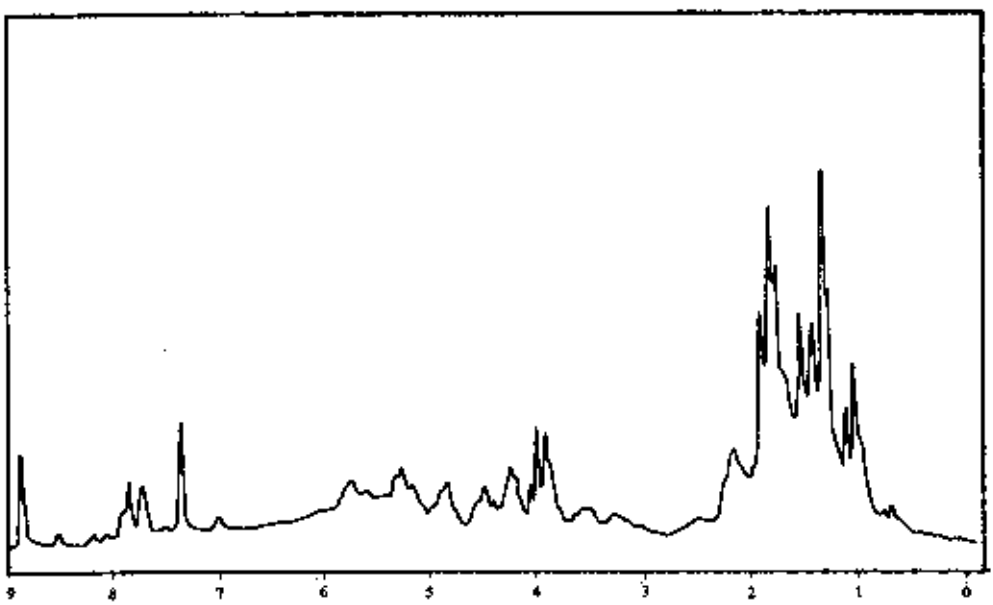

Fig. 3. PMR spectrum of Holothurin B of H. pulla.

LIFE HISTORY OF *HYDROCLATHRUS CLATHRATUS* (BORY) HOWE (SCYTOSIPHONALES, PHAEOPHYCEAE) IN LABORATORY CULTURE

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ABSTRACT

This study describes the life history of *Hydroclathrus clathratus* in laboratory culture. Liberated swarmers from plurilocular sporangia were cultured at different temperature-daylength conditions using modified Provasoli's enriched medium. Through either a filamentous or discoid type of early germination, the germlings developed asexually. Short daylengths induced the formation of unilocular sporangia on crust plants and enhanced the development of plantlets into globular thalli; whereas, long daylenghts favored the growth of clathrate thalli. Swarmers released from plurilocular sporangia either developed directly into *Hydroclathrus* plants similar to the wild plants, or into crust plants with unilocular sporangia which later released swarmers that finally grew into *Hydroclathrus* thalli like those found in the field.

INTRODUCTION

The order Scytosiphonales was established by Feldmann in 1949 and three families, Phaeosaccionaceae, Scytosiphonaceae and Chnoosporaceae were included. The genus *Hydroclathrus* which belongs to the family Scytosiphonaceae was established by Bory in 1826. Originally, it was known as *Encoelium clathratum* Agardh (1822) and different taxonomic names were given to the alga; *Hydroclathrus cancellatus* Bory, *Fucus clathratus* Bory, *Stilophora clathrata* Ag., *Asperococcus cancellatus* Endlicher, *Halodictyon cancellatum* Kutz., and *Asperococcus clathratus* J. Ag. It was restudied by Howe in 1920 and since then, has been named *Hydroclathrus clathratus* (Bory) Howe.

The only known species of the genus was *Hydroclathrus clathratus* (Setchell and Gardner, 1925, Okamura, 1936), until 1983 when Tseng added another species, *H. tenuis* Tseng *et* Lu. It differs from *H. clathratus* by its thin and fine reticulate frond. As reported by Okamura (1936), *H. clathratus* thallus is irregular in form and adheres by root-like hairs to rocks and stones near or below low tide mark; cortical portion made up of one layer of pigmented cells and medullary portion consists of large colorless cells; spores produced in plurilocular sporangia. *H. clathratus* is chiefly distributed in tropical and sub-tropical waters or sometimes in the temperate zone. In the Philippine islands, it is commonly found in Luzon, Visayas and Mindanao. In Japan, the species is known from Honshu, Shikoku, Kyushu and the southwestern Islands. The materials in this study were collected at Kodomari Cove in Takahama Bay, a branch bay of Wakasa Bay where they grow abundantly in spring and early summer.

Studies on Scytosiphonaceae have included complete life history, temperature-daylength relationships in laboratory culture to phenology in the field. Conjugation of gametes in *S. lomentaria* by Frye (1930) and *Colpomenia sinuosa* by Kunieda and Suto (1938) gave initial results which paved the way to further investigations on the life history of the members of Scytosiphonales. Dangeard (1963) first observed that microscopic crust thalli in *S. lomentaria* and *Petalonia fascia* bear unilocular sporangia. The same observation was made by Tatewaki (1966), Lund (1966a and b), Wynne (1969), Hsiao (1969), Edelstein *et al* (1970), Nakamura and Tatewaki (1975), and Clayton (1976a). Likewise, unilocular sporangia were found in the sporophytes (microthalli) of *Colpomenia bullosa* and *Endarachne binghamiae* (Nakamura and Tatewaki 1975). The influence of light quality on the development and fertility of macrothallus in *Petalonia* and *Scytosiphon* was studied by Luning and Dring (1973). Clayton reported the effects of season, temperature, and daylength on *S. lomentaria* (1976b) and factors controlling sexual reproduction in *Colpomenia peregrina* and *Scytosiphon* sp. (1981). The only known study on the life history of *H. clathratus* was made by Clayton (1982); however, there is paucity on its description. She describes a "direct" life history of *H. clathratus* and some known brown algal species (*Chnoospora implexa, Colpomenia sinuosa, Endarachne binghamiae, Petalonia fascia, Asperococcus turneri* and *Punctaria latifolia*). Two alternating generations (one a large polystichous macrothallus with plurilocular sporangia and the other a haplostichous ralfsioid microthallus with unilocular sporangia) were observed in several Scytosiphonaceous species in Japan (Nakamura and Tatewaki 1975). Since there were no conjugating gametes observed in *H. clathratus*, the term swarmer released by the plurilocular and unilocular sporangia is adopted in this paper. Further study of this species as to its sexual reproduction is thus encouraged.

There are limited reports on the utilization of *H. clathratus* in the world. Among the tropical countries in Southeast Asia, Philippines and Malaysia primarily utilized it as human food in the form of salad (Galutira and Velasquez, 1963 and Shiew-Moi, 1984) and as a source of alginic acid (Shiew-Moi, 1984). Studies of this species center mainly on taxonomy and morphology. This investigation was made to establish the life history of *Hydroclathrus clathratus*, an area of study still undocumented to date. This new knowledge will furnish baseline information for possible sea farming and expansion of its utilization.

MATERIALS AND METHODS

Algal specimens of *Hydroclathrus clathratus* were collected at Kodomari Cove, Wakasa Bay, middle part of Honshu facing the Japan Sea (Figs. 1A and B) on June 10 and July 8, 1983.

Thalli were first examined under the microscope to determine the presence of reproductive structures. Each frond was fragmented and rinsed several times with autoclaved seawater and both sides were brushed with a camel's hair brush. These were air-dried for an hour and placed in separate Petri dishes containing sterilized seawater. An illumination of 3000-5000 lux at 20°C with daylength of 16:8h provided the laboratory condition for the liberation of swarmers. These were pipetted on glass slides and cultured

at different temperature-daylength conditions. Medium used was modified Provasoli's enriched seawater (ESI, Tatewaki, 1966) and was changed fortnightly. Five milligrams of GeO_2 per liter was added to the medium to kill diatoms in early cultures (Lewin, 1966).

Temperature-daylength conditions used

I.	10°C 10:14h	IV.	15°C 14:10h
II.	10°C 14: 10h	V.	20°C 10:14h
III	15°C 10:14h	VI.	20°C 16:8h

VII. 25°C 14:10h

RESULTS AND DISCUSSION

There were 2 types of life histories observed: (1) an alternation of two generations – a macrothallus with plurilocular sporangia (*Hydroclathrus* phase) and a microthallus (crust plant) bearing unilocular sporangia; and (2) the direct development of swarmers into *Hydroclathrus* plants.

1. Alternation of two generations

1.1 Development of swarmers from plurilocular sporangia

Fertile plants from nature bore plurilocular sporangia in sori. The sporangia were uniseriate to mostly biseriate, clavate in shape, possessing 5-10 compartments and measuring 16.0-48.2 μm x 6.0 – 8.4 μm ($\bar{X}$ = 24.8 μm x 7.6 μm) in size (Fig. 2A).

At condition VI, swarmers from a single plant were released. They appeared to be pear-shaped, 4.0-10.0 μm x 3.0 – 5.0 μm ($\bar{X}$ =5.0 x 4.0 μm), possessing a plastid with a pyrenoid (Fig. 2B) and two lateral flagella. The anterior flagellum had a maximum length of 13.2 μm and the posterior, a maximum length of 6.3 μm. Swarmers tended to swim away from the source of light (negative phototaxis). Their motility lasted for more than a day, then they settled at the bottom of the glass slide becoming spherical, measuring 3.6 – 8.0 μm (X = 4.9 μm) in diameter and possessing a single eyespot (Fig. 2C). Those cultured in individual test tubes at condition VI had their germlings settling on the walls away from the light. Before germination, the settled swarmer grew to 10.0 μm in diameter. After 2–3 days, the settled swarmer began to germinate by pushing out a protuberance. The original cell retained its protoplast (Fig. 2D). Two types of development were observed at condition VI, namely:

1.1.1 Filamentous type – By one transverse division the cell divided into two (Fig. 2E). Further divisions took place, producing a uniseriate filament consisting of four cells (Fig. 2F), then a 6-celled filament which was provided with a hair apically (Fig. 2G) after 6 days. The uniseriate filament produced prostrate lateral branches (Fig. 2H) after 7-12 days, formed upheaved cells at the center (Fig. 2I) and finally coalesced to form disc-like plantlets 0.3-0.5 mm in diameter after 15-25 days (P1.1–B).

1.1.2. Discoid type – The settled swarmer germinated to form 2-6 lobes with a diameter of 18.0 – 28.0 μm (Figs. 2J-L) and after 7-10 days formed a disc by radiate formation, measuring 60.0 x 60.0 μm. The peripheral cells of the disc were characteristically Y-shaped (Fig. 2M).

Ten to fifteen-day-old germlings from VI were transferred to the following conditions: I, II, III, IV and V. Cultures kept at I and II remained sterile for 6 months. Those kept at IV and VI produced elongated unilocular sporangia after 85-97 days, however, 90-95% of their germlings were bleached. Those which were kept at III and V grew well and became fertile, bearing unilocular sporangia after 77 and 68 days respectively.

Unilocular sporangia, obovoid-ovoid in shape, measuring 20.0 – 100.0 μm x 20.0 – 62.0 μm ($\bar{X}$ = 46.0 μm x 30.0 μm) in size (Fig. 3A), were produced terminally on the erect filaments of the discoid thallus.

1.2 Development of swarmers from the unilocular sporangia

Swarmers liberated at conditions III and V were pear shaped, 4.0 – 6.0 μm x 3.6 – 4.0 μm (Fig. 3B), provided with a single eyespot and a plastid with two lateral flagella, the anterior being longer (10.0 – 16.0 μm) than the posterior (4.0 – 6.0 μm). There was no observed fusion of swarmers. Upon settlement, they became spherical, measuring 4.0 – 10.0 μm ($\bar{X}$ = 5.6 μm) in diameter (Fig. 3C). After a day or two, they began to germinate by pushing out a protuberance (Figs. 3D-E) and later divided transversely into two cells (Fig. 3F). By successive cell divisions, the two celled germling became a uniseriate filament after 5-6 days and a hair was observed apically (Fig. 3G). After 10-15 days, this formed branches uprightly (Fig. 3H). By successive cell divisions and branchings, there were densing and upheaving of the cells at the center of the plantlet which measured 160.0 μm in diameter (Fig. 3I). Growth increased rapidly in size until finally the plantlet became a globular *Hydroclathrus* thalli measuring 0.5 – 1.0 mm in diameter after 30-40 days. On the other hand, swarmers germinated and developed into minute discs (crusts) consisting of 3-5 cells and measuring 30.0 – 50.0 μm in diameter (Figs. 3J-L). They increased their size by successive transverse and longtudinal cell divisions (Figs, 3M-N) and finally grew into globular *Hydroclathrus,* measuring 0.6 – 1.0 mm in diameter after 30-40 days (Pl. 1-H).

Plants cultured at III and V reached maturity in 165 to 177 days. Mature thallus, 3.0 – 6.0 mm in diameter which had an entirely smooth surface, subsequently produced sori bearing clavate plurilocular sporangia which were uniseriate-biseriate, measuring 22.0 – 30.0 μm x 6.0 – 14.0 μm ($\bar{X}$ = 25.0 μm x 9.0 μm) in size (Pl. 2A-B). Appearance of small perforations on the surface of the thallus was observed on the 30th – 35th day of culture (Pl. 2-C). Cultures was observed a typical clathrate thalli after 45-55 days (Pl. 2D). Though the thallus had perforations, it released swarmers two to three times at intervals of 25-35 days.

When crust plants at VI bearing immature unilocular sporangia were transferred to V, they matured and released swarmers which germinated and developed into globular plants after 32 days. Thirty to thirty-five day-old cultures at III and V were transferred to the following conditions: VII, VI, IV and I. Some cultures at VII were bleached after 61

days; those which survived were later transferred to VI. All cultures at condition I were bleached after 55 days. Clathrate thalli were observed at VI, V, IV and III; however, those cultures in long day conditions IV and VI were darker brown in color than those in short day conditions (I and III).

A transverse section of the cultured thallus revealed two distinct layers: 1) an outer layer – measuring 3.0 – 10.0 μm ($\bar{X}$ = 7.0 μm) and consisting of cuboidal cells each containing a plastid and a pyrenoid; 2) an inner layer – measuring 50.0-310.0 μm ($\bar{X}$ = 146.0 μm), colorless and with polygonal cells (Pl. 2E). Cultured specimens closely resembled wild plants.

2. Direct development of swarmers from plurilocular sporangia

Plurilocular sporangia in sori from nature were uniseriate to biseriate, measuring 20.0 μm x 4.0 μm with 2-4 rows of compartments (Fig. 4A).

Actual release of swarmers at condition VI was not observed. However, settled swarmers were found to be spherical in shape measuring 3.6 – 7.2 μm in diameter, provided with a single eyespot and a parietal plastid (Fig. 4B). Three days later, a protuberance 15.0 μm x 7.0 μm in size was observed pushing out from the cell (Fig. 4C). The cell later transversely divided into two, attaining a size of 20.0 μm x 6.0 μm (Fig. 4D). Further transverse divisions took place producing a uniseriate filament 30.0 μm in length consisting of 4 cells after 4-6 days. This later formed upright branches attaining a length of 131.0 μm after 7-10 days (Fig. 4F). By longitudinal and transverse cell divisions, the branched filament formed upheaved cells at the center of the plantlet (Fig. 4G). Sixty-three days later, globular plantlets less than a millimeter in size were observed (Pl. 3A). However, few filamentous plantlets remained as crusts. At this stage, some globular plantlets were transferred to conditions I, III, IV, V and the rest remained at VI. After 69 days, plants at I measured 2.0 – 3.0 mm ; those which remained at IV measured 1.8 – 3.3 mm; those which were transferred from III to VI measured 2.6 – 3.7 mm after 69 days; and finally those which came from V measured 3.5 – 4.8 mm after 71 days and were later cultured at VII. Several phaeophycean hairs were seen in each thallus and they were 1.0 – 13.0 mm long. When young globular thalli were transferred from conditions I to VI and from III to VI, their maturity, i.e. the presence of plurilocular sporangia, was attained after 78 days; those kept at IV matured after 42 days; when those kept at V were transferred to VII they attained maturity and a size of 2.0 – 6.0 mm (Pl.3B-C) after 71 days. Clathrate thalli of the species were found 120-150 days after maturity in VI and IV (Pl.3D). Unfortunately, the thalli were found to be bleached at condition VII on the 159th day of culture.

External and internal morphologies of the *H. clathratus* plants which developed directly from swarmers bore similarities (Pl.3E) with those developed from swarmers released by unilocular sporangia formed on microthalli (crust plants).

Clathrate *Hydroclathrus* which developed directly from swarmers of plurilocular sporangia bore resemblances with those found in nature.

Clayton (1982) gave the first report on the "direct" life history of *Hydroclathrus clathratus*. She showed that zooids released by macrothalli do not behave as gametes and most of them developed directly into similar macrothalli. The present study also obtained information regarding a direct life history.

H. clathratus is known to be a seasonal species, i.e. it is only present in nature within a certain season and absent for the rest of the year. Irregular globular thalli of this species bearing plurilocular sporangia were found in Kodomari Cove during the months of April to July in 1983, when the average surface water temperature ranged from 13.1 – 22.6°C and daylengths of 13-15:9-11h. Highest surface water temperatures were recorded during the months of August to September and the lowest, 10°C in February (Fig. 1C). The early spring appearance of *H. clathratus* in the coast of Kodomari Cove can be correlated with the requirement of low temperature and short daylengths for the development of the irregular globular thallus from the swarmers released by the microthalli. There were collections of fertile *H. clathratus* in Victoria and Queensland, Australia by Clayton (1982) in June 1976 and 1977 (early winter, 9.5:14.5h), October 1975 (spring, 12.5:11.5h) and November 1976 and 1977 (summer). There was no collection of macrothallus during autumn. During the senior author's personal visits to Shirahama and Kushimoto, middle part of Honshu, facing the Pacific Ocean, this species was found in abundance from January to April. Temperature in these areas is slightly higher than in Kodomari Cove during these the macrothallus starts to appear from late December (Galutira and Velesquez, 1963) to May (Velasquez *et al* 1971). There was, however, a collection in Eastern Samar during the month of November by Cordero (1980). There are only two seasons in this country (dry and west). Distribution of rainfall differs geographically from north to south and from east to west. Also, there are two pronounced daylengths, 11:13h (November to February) and 13:11h (April to September). Based on the preceding observations in these places in Australia, Japan, and the Philippines, macrothalli occurrence ranges from early winter to summer time and microthalli production is assumed to be during the late summer to autumn.

Dimorphism was exhibited by this species during early swarmer germination in both generations: 1) filamentous and 2) discoid. In the filamentous type, the settled swarmer developed a protuberance which later became filamentous and finally developed into a prostate system from which an irregular globular thalli developed. This type of development was also observed in *Scytosiphon lomentaria* (Tatewaki, 1966; Nakamura and Tatewaki, 1975; Pedersen, 1980 and Clayton, 1980), in *Petalonia fascia* (Wynne, 1969; Nakamura and Tatewaki, 1975), and in *P. zosterifolia, Colpomenia bullosa* and *Endarachne binghamiae* (Nakamura and Tatewaki, 1975). Discoid type of germination was observed by Wynne (1969) in *E. binghamiae*. Clayton (1981) observed settled swarmers of *C. peregrina* developing directly into small, spherical balls of cells which became hollow at an early stage in their growth. According to Pedersen (1980), stellate (discoid) germination is certainly genetically determined, but in some cases, it seems to be triggered by contact with the substratum. Differences in germination seem to be a morphological response to various types of substrata but Hsiao (1970) positively concluded on *Petalonia fascia* that there is a combined effect of light intensity and temperature on growth, morphology, and reproduction. This was further confirmed by his earlier report (1969) on the same species about iodine concentration appearing to be essential in its morphogenesis.

The observations of Wynne (1969), Edwards (1969), Rhodes and Connell (1973), Roeleveld *et al* (1974) and Nakamura and Tatewaki (1975) in *P. fascia* to produce erect thalli at 15° and 20°C short day conditions parallel our results (III and V). This phenmenon has also been observed in *Scytosiphon lomentaria* (cylindrical form) by Tatewaki (1966), and Wynne (1969) and in the complanate form by Clayton (1976a). Geographical distribution of *S. lomentaria* results in different daylength responses as reviewed by Lüning (1980).

Germlings at long day conditions (IV and VI) either died during the early stage or remained sterile as discoid or filamentous plantlets. This observation resembles the findings of Rhodes and Connell (1973) in *P. fascia*, that most microthalli (greater than 90%) produced blades at 10° and 21°C under 9:15h daylength but few microthalli (less than 1%) produced blades at 21°C under 14:10h daylength. Earlier reports of Wynne (1969) on *P. fascia* agree with this study that warm temperature and long daylength or both may kill germlings that would produce blades resulting in a preponderance of crusts. This is in contrast to Clayton's (1979) findings on *C. peregrina*, that saccate plants were produced in all daylengths tested (8:16h, 11:13h, and 16:8h at 16°C).

Change from long (IV and VI) to short (III and V) daylength conditions induced the formation and maturation of unilocular sporangia as well as development of globular thalli. This confirms earlier studies of Tatewaki (1966) and Nakamura and Tatewaki (1975). Clayton (1976b) in her study of *S. lomentaria* in southern Australia, concluded that daylength has not been observed to directly alter the essential morphological form (destined form) of a plant, a germling, or even a zooid. Her results imply that morphogenetic stimulus is only effective before or during spore formation.

The induction of erect thallus may require low irradiance and the subsequent formation of the erect thallus requires sufficient light to support photosynthesis (Lüning, 1980). This probably explains why *Hydroclathrus* plants became clathrate during long daylengths under both laboratory and field conditions.

The life history of *Hydroclathrus* manifests the same features as the other members of Scytosiphonales. In this study, *H. clathratus* perpetuated asexually like *P. fascia* (Wynne, 1969). Due to temperature-photoperiod response such life cycle occurred. Some species of Scytosiphonales reproduce both asexually and sexually or only asexually. Geographical location and season are variables affecting reproduction. Tatewaki (1966) and Nakamura and Tatewaki (1975) claim *S. lomentaria* in Hokkaido, Japan and *E. binghamiae* in Chiba, Japan, reproduce both asexually and sexually; whereas Wynne (1969) observed that these two species in California, North American perpetuate asexually. Wynne's conclusion on *S. lomentaria* was further confirmed by Rhodes and Connell (1973) on the Atlantic Coast of Virginia. Complanate form of *S. lomentaria* in southern Australia has been reported by Clayton (1976a) to reproduce sexually, but Pedersen (1980) asserted its asexual generation in West Greenland. *S. dotyi* (Wynne, 1969) and *P. fascia* (Wynne, 1969; Nakamura and Tatewaki, 1975) are known to reproduce asexually. *C. bullosa* (Nakamura and Tatewaki, 1975) and *C. peregrina* (Clayton, 1979) are reported to propagate sexually and asexually. A continuing study on the life history of *H. clathratus* is imperative.

SUMMARY AND CONCLUSIONS

1. There are 2 types of early swarmer germination known as heteroblasty in both generations of the heteromorphic life history: a) filamentous germination, wherein settled swarmer developed a protuberance which later became filamentous and finally developed into a prostrate system from which an irregular globular thalli developed, b) discoid germination, wherein the settled swarmer formed 2-6 lobes which later formed a disc by radiate formation with a characteristic Y-shaped cells along the margin.

2. Short daylength induced the formation of unilocular sporangia on microthallus and the development into globular thalli, while long daylength favored the growth of clathrate thalli.

3. Swarmers released from plurilocular sporangia either developed directly into macrothalli similar to the wild plant (direct life history) or into microthalli with unilocular sporangia which released swarmers which finally grew into macrothalli like the *Hydroclathrus* plants found in the field (heteromorphic life history).

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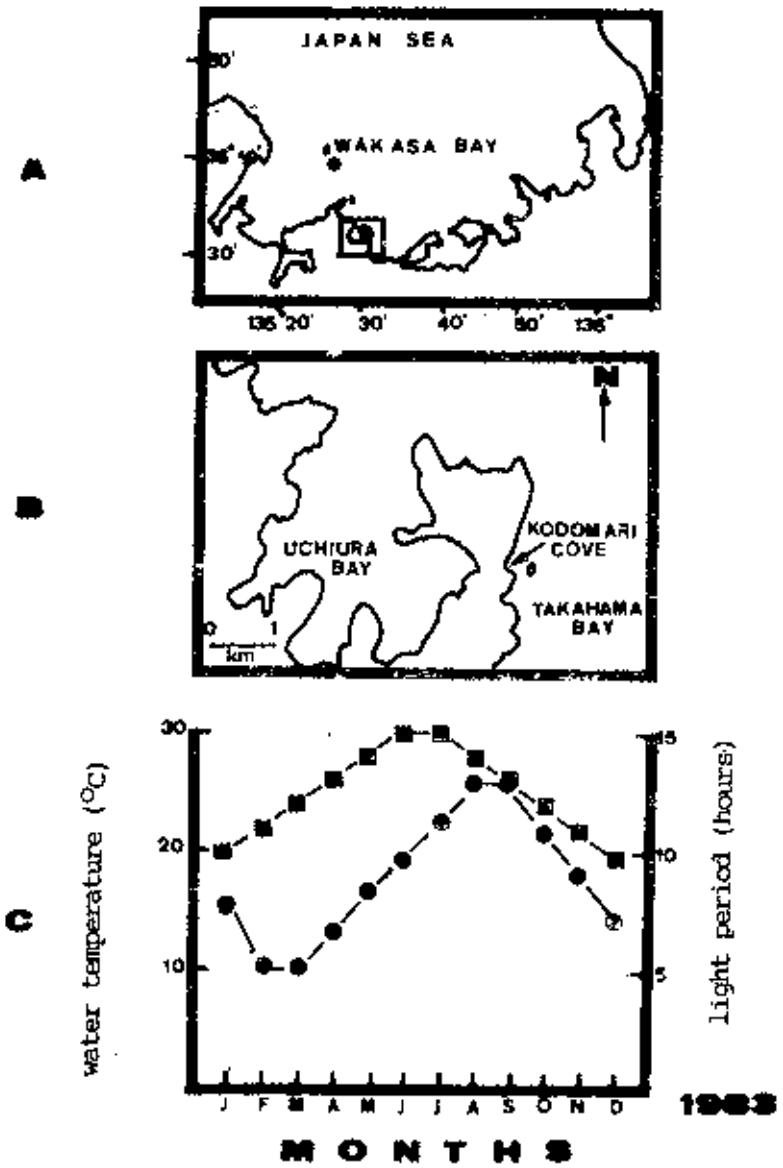


Fig. 1A. Map of Wakasa Bay and its branch bay, Takahama Bay. (□) B. Map showing Kodomari Cove (arrow). C. Average monthly surface water temperature and daylength at Keshimo (Wakasa Bay). Data obtained from Kyoto Institute of Oceanic and Fishery Science. o temperature; o daylength.*

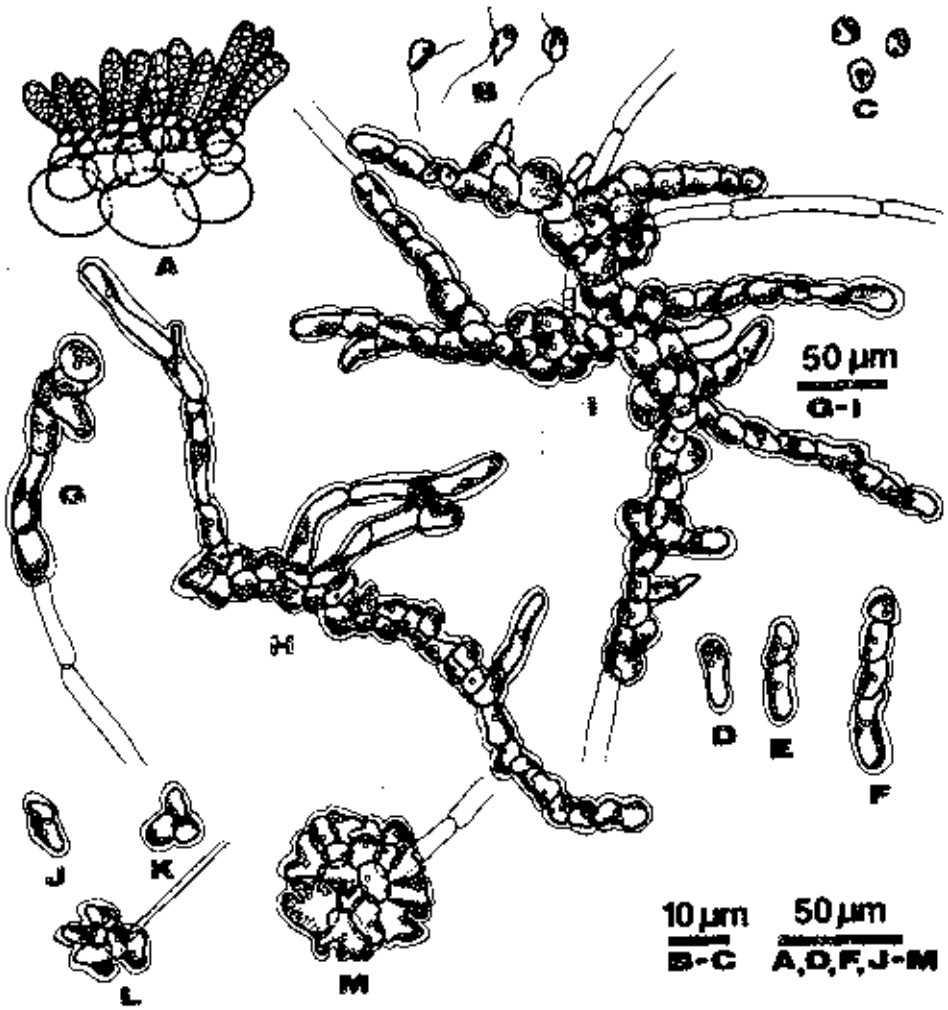


Fig. 2. Development of swarmers from plurilocular sporangia: A. section of a part of macrothallus from the field showing the plurilocular sporangia. B. swarmers.; C. settled swarmers. D. germination of swarmer. E. 2-celled germling. F. 4-celled germling. G. uniseriate filamentous, 6-day-old germling. H. 12-day-old germling. I. filamentous plantlet with prostrate branches. J-L. 2-6 lobed germlings. M. minute disc plantlet.

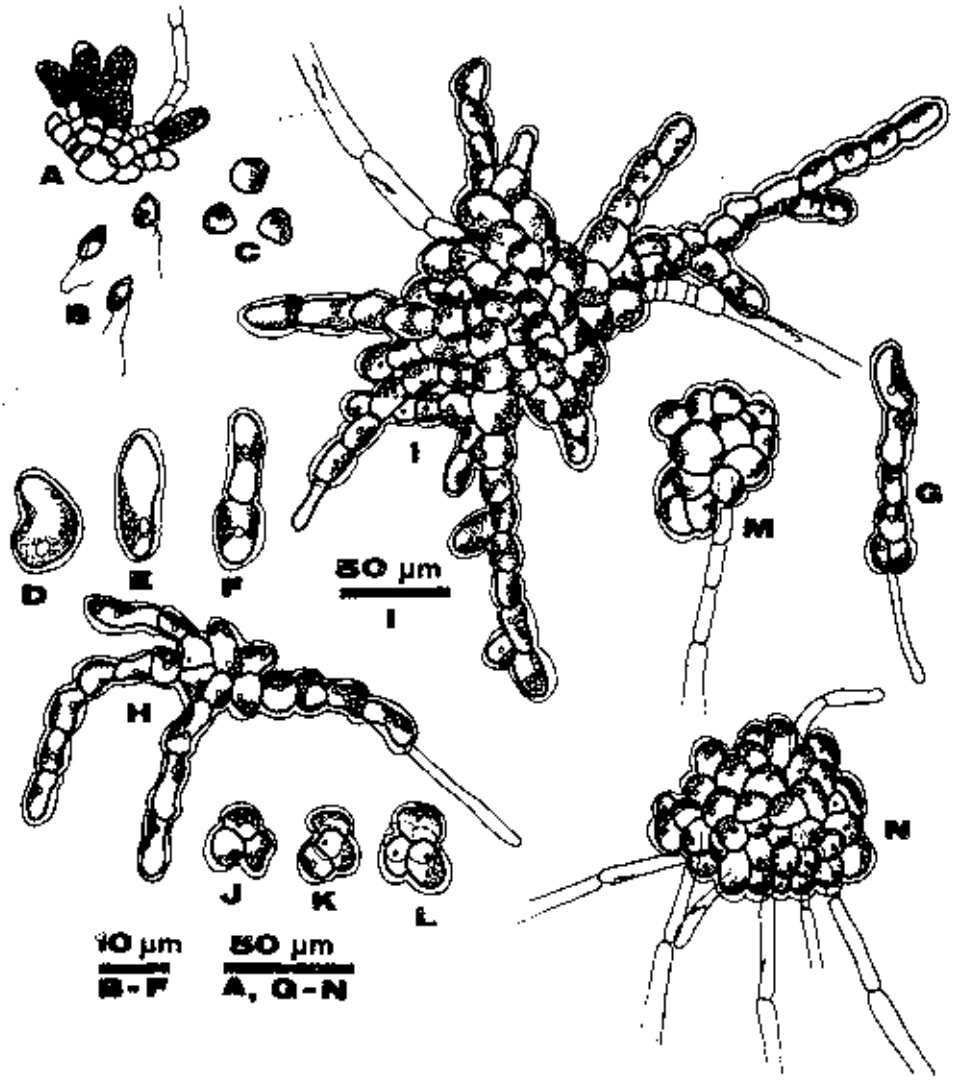


Fig. 3. Development of swarmers from unilocular sporangia: A. section of a part of a microthallus with unilocular sporangia. B. swarmers. C. settled swarmers. D-E. germination of swarmer. F. 2-celled germling. G. 5-day-old germling. H. branched-filamentous plantlet. I. plantlet with upheaved cells at center. J-L. 3-lobed germlings. M-N. young minute disc-plantlets.

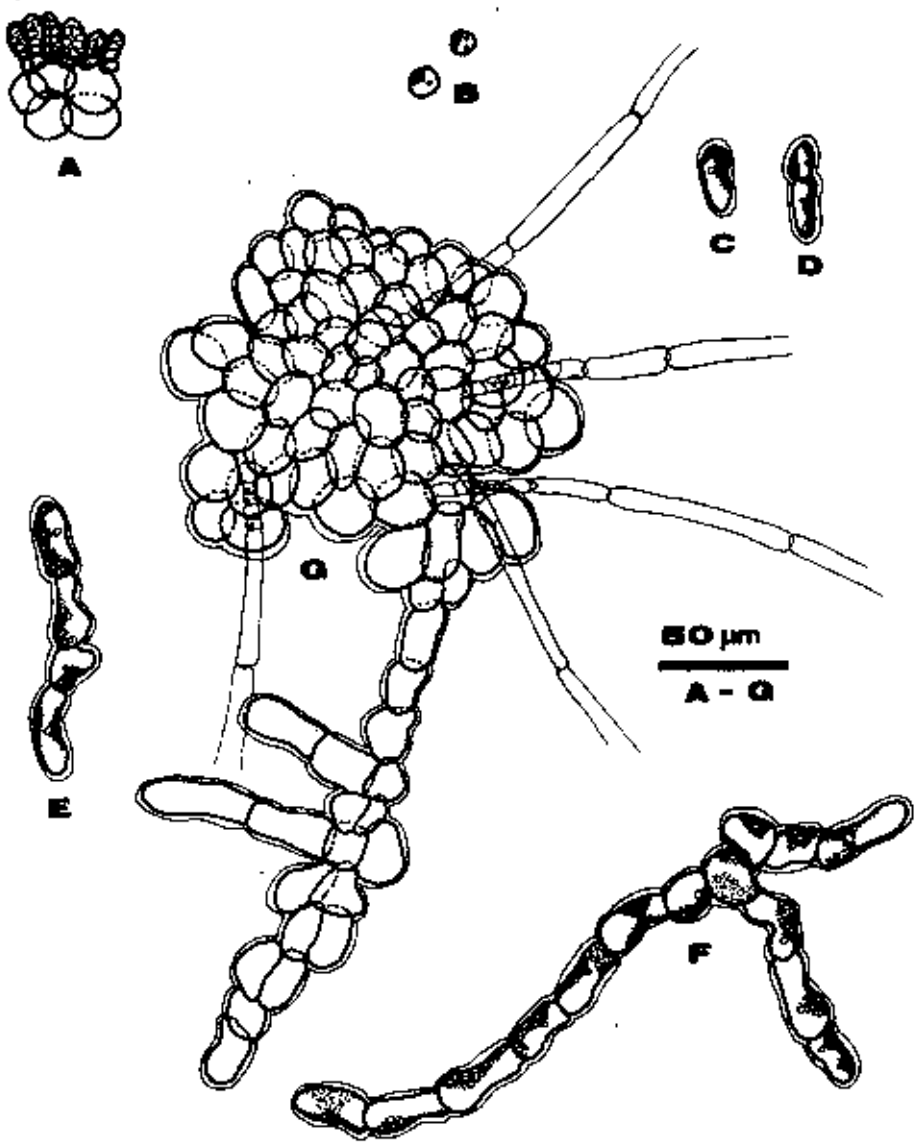


Fig. 4. Direct development of swarmer. A. section of a part of macrothallus from the field with plurilocular sporangia. B. settled swarmers. C. germination of swarmers. D. 2-celled germling. E. 4-celled germling. F. uniseriate branched filament, 10-day-old germling. G. formation of globular thallus from upheaved cells of the plantlet.

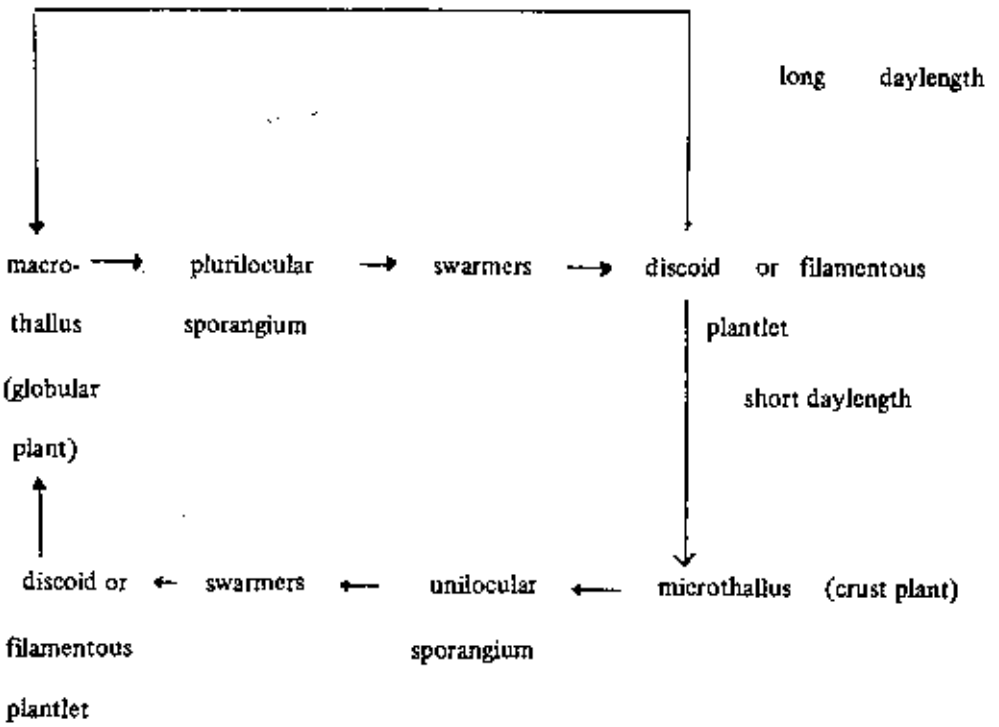
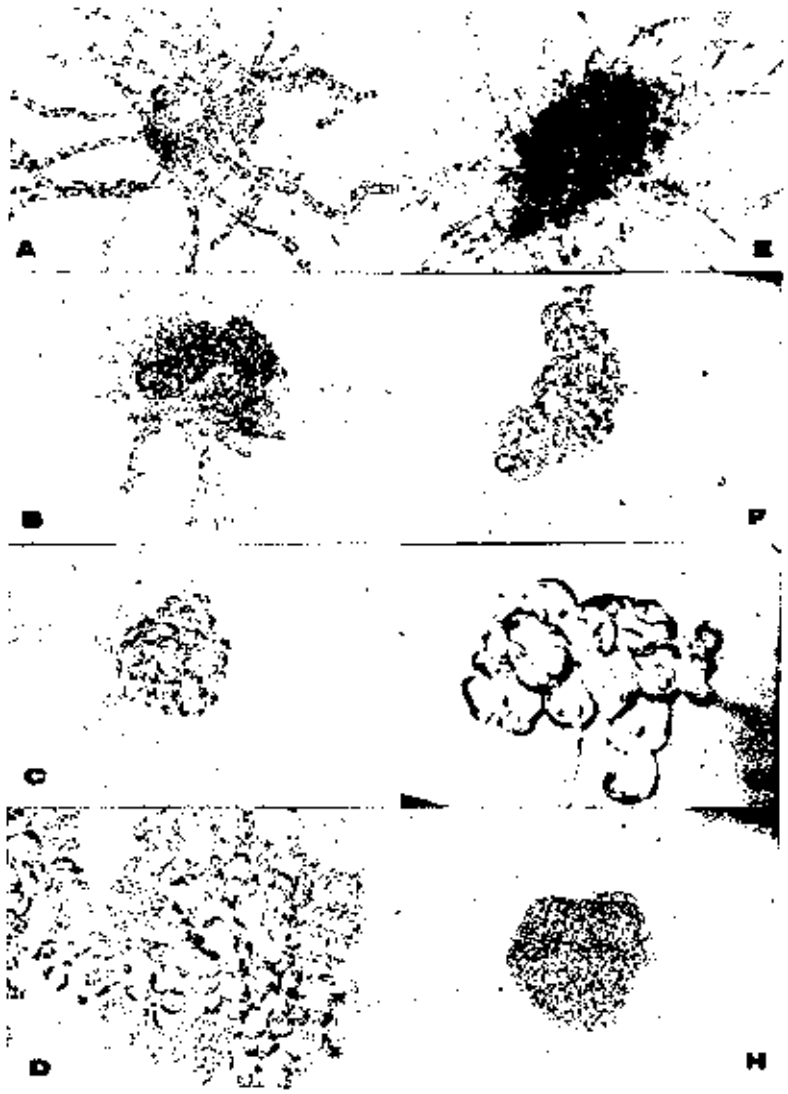


Fig. 5. A schematic illustration of the life history of Hydroclathrus clathratus.

Plate 1

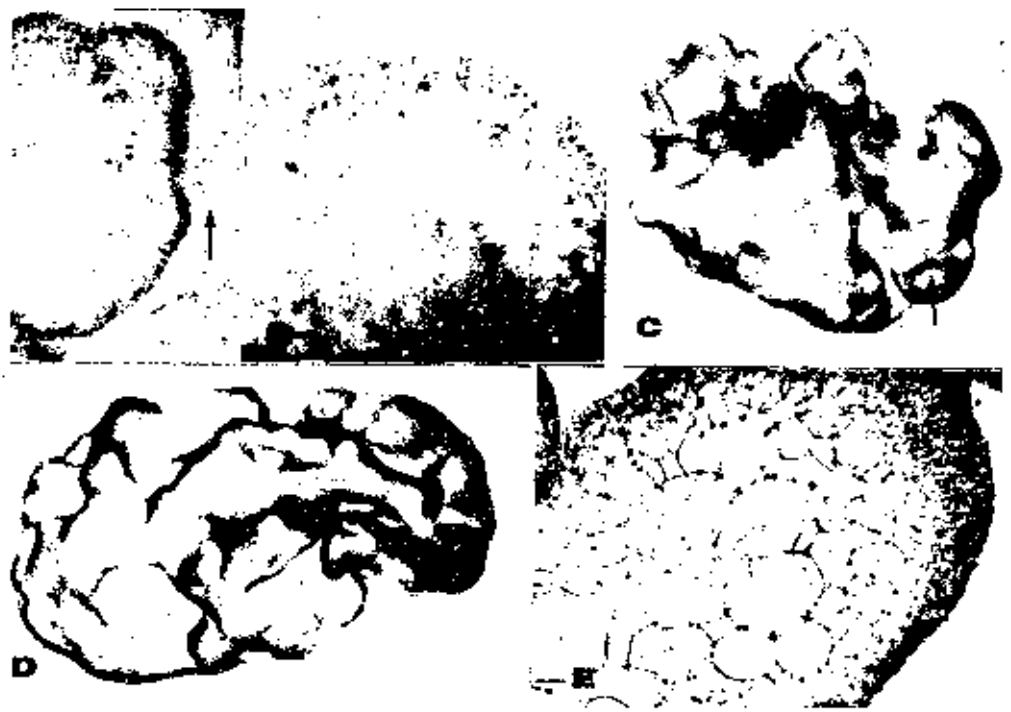


A.-D. Development of thallus from swarmers of unilocular sporangia.

A. formation of upheaved cells at center.
B. dense upheaved cells, several hairs arising from the center.
C. disc-like plantlet, provided with several hairs.
D. squash preparation of microthallus bearing obovoid unilocular sporangia.

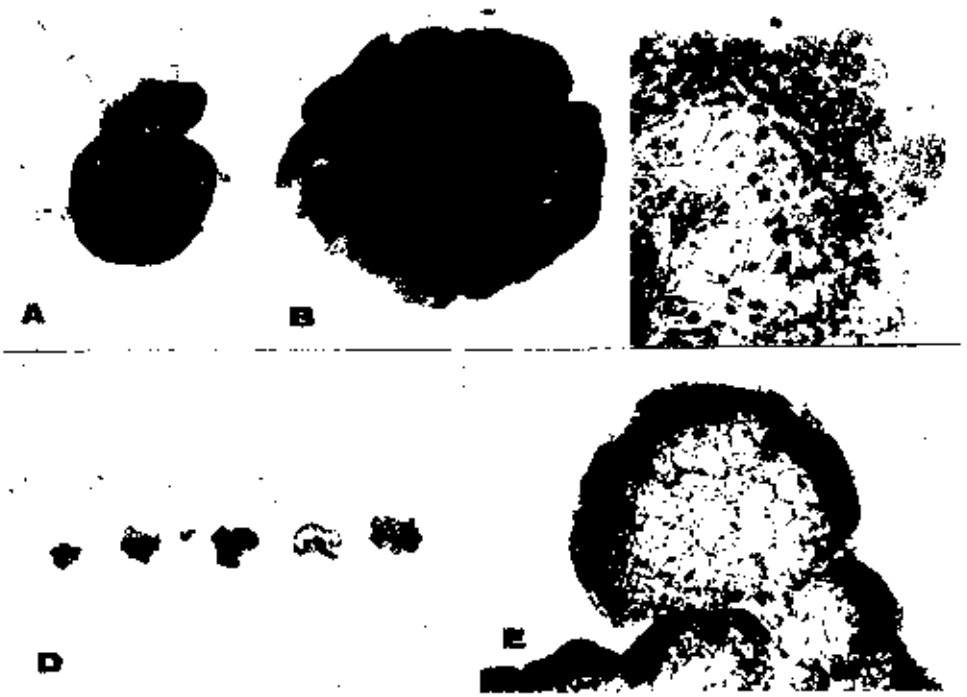
E-H. Development of thallus from swarmers of unilocular sporangia.
E. plantlet with dense upheaved cells at center.
F. globular formation of the thallus.
G. disc-like plantlet with hairs.
H. young globular thallus.

Plate 2



A-E. Development of thallus from swarmers of unilocular sporangia.
A. pluriocular sporangia in sorus (arrow)
B. detailed pluriocular sporangia
C. globular thallus with small perforations (arrow).
D. clathrate thalli.
E. transverse section of the thallus with two distinct layers.

Plate 3



A-E. Direct development of swarmers from plurilocular sporangia
A. 63-day-old globular thallus
B. globular thallus growth at 15°C 14:10h (134-day-old)
C. squash preparation of a portion of a matured thallus bearing plurilocular sporangia
D. clathrate thalli grown at 15°C 14:10h
E. transverse section of the globular thallus showing the outer cortical layer and inner medullary layer.

TWO ORB-WEAVING SPIDERS (ARANEAE: ARANEIDAE) IN THE PHILIPPINES CAUSING ARANEIDISM*

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ABSTRACT

The systematic descriptions, illustrations and photographs of two species of orb-weaving spiders, *Nephila pilipes* (Fabricius) and *Neoscona mínima* F.O.P. that caused araneidism in the Philippines are presented.

INTRODUCTION

Spiders are obligate predators of insects and mites found in a wide range of habitats in the tropics. With the current universal concern for environmental quality, they are being considered in pest management programs as one of the prospective biological agents against major insect pests in agricultural crops. The spider fauna of the Philippines consists of a wide array of species. For instance, in the rice agro-ecosystems, the survey of the spider fauna revealed 57 taxa consisting of 34 genera under 16 families (Barrion, 1980).

Generally, spiders are very shy and timid creatures, but, when disturbed or attacked by their natural enemies, like man, their immediate means of defense is through their bites. During the nineteenth century, the danger of a bite from a spider was widely underestimated and even denied. More precise knowledge and critical observations have, however, shown that the venom of a limited number of spiders has serious effects which are not shared by the others (Savory, 1964). The poisoning caused by the bite or sting of a spider is called araneidism. So far as is known, with the exception of the members of two small families, all spiders have poison glands (Hunter, 1966). The venom apparatus consists of a pair of glands in the cephalothorax specifically in the basal segment of each chelicerae from which a duct leads to a small opening near the tip of the chelicerae of the same side. This opening is so placed that it is not closed by the pressure of the bite but allows the venom to flow into the wound. The emission of the venom is apparently under voluntary control and its virulence varies in different species. The quantity of the venom ejected vary with the inclination, age and physiological condition of the spider. It also depends upon the degree of irritation to which the spider was subject (Kaston, 1948).

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In 1985, two separate cases from Los Baños, Laguna were brought to the attention of the senior author by a mother and an aunt of two boy victims of araneidism. Both boys complained that they were bitten on their hands by the spiders while they were climbing the mango trees. The immediate manifestations of the spider bites were localized swellings on bitten arms, high fever and contractions of the leg muscles. Later, there were observed necrosis and gangrenous effects on the bitten areas. Fortunately, the boys recovered after two months of continuous intake of analgesic and antipyretic medicine plus antibiotics.

On June, 1986, another case of araneidism was reported by Dr. Patricia Grace E. Dañgo whose patients were children bitten by a spider in Cabanatuan City. The spider is popularly known as "gagambang gubat" and the children fondly use it as a game animal. The patients exhibited contraction of leg muscles, high fever, infected kidney and liver, jaundice and hemoglobinuria.

The reported manifestations and clinical results of araneidism in the Philippines coincided with the earlier reported cases in other countries.

In America and Australia, the venom of the black widow, *Latrodectus mactans* (Fabricius), is neurotoxic, the muscular tonus heightened giving rise to painful rigidity of the abdominal wall, contraction of the intercostal muscles with a feeling "tightness" of the chest and contraction of leg muscles (Smith and Russell, 1967). Acute symptoms which persisted for 12 to 48 hours included increased temperature and blood pressure, spinal fluid pressure, exaggeration of reflexes, leucocytosis and often profuse perspiration, priapism, nausea and localized edema. Death resulted from 14 to 32 hours from asphyxia preceded by convulsions (Kaston, 1948).

The treatment involving intravenous administration of 10 mL of 10 per cent calcium gluconate relieved the victims from muscle pain and spasms from 30 minutes to 4 hours. If relief of muscle spasm and pain is not obtained with calcium gluconate, skeletal muscle relaxant drugs such as methocarbamol or mephesin provide adequate control for 8 to 16 hours when given in continuous dosage. They relieved the patient's headache, nausea and respiratory distress. A specific antivenin prepared from the serum of horses hyperimmunized to the venom relieves the symptoms and speeds recovery from *Latrodectus* poisoning (Hunter, 1966).

In Taranto, South Italy, the bite of a wolf spider, *Lycosa tarentula* (Rossi), led to death through paralysis of respiratory muscles (Kaston, 1948).

In the Americas, the venom of *L. tarentula* had little harmful effect on man but the powerful chelicerae of large species are capable of producing painful wounds. The large black tarantula, *Sericopelma communis* Cambridge was reported to be poisonous in the Canal Zone (Hunter, 1966).

The venom of South American *L. raptoria* (Walckenaer) caused extensive injuries to the skin and neighboring muscles (Kaston, 1948).

A wolf spider, *Scaptocosa raptoria* (Walckenaer) was reported to have a potent hemolytic venom and has been incriminated in necrotic araneidism in Brazil (Vellard, 1936).

The venom of an orb-weaver, *Mastophora (Glyptocranium) gasteracanthoides* Nicolet, caused jaundice and hemoglobinuria resulting in death of vineyard workers after 5 or 6 days. Fatal cases have been known in which the patient bitten in the throat died due to suffocation and septicaemia. The type of venom which injured the kidney and liver caused a necrosis of tissues in the region of the bite and took from 6 to 10 days to heal (Kaston, 1948).

In Chile and other parts of South America, Central America and Guatemala, the six-eyed false hackled-band spinner, *Loxosceles laeta* (Nicolet), caused cutaneous necrosis, erythema, edema and formation of a blister which after rupture became gangrenous. A severe systematic reaction including a fatal hemolytic anemia may follow envenomation (Frawley, 1935).

Besides the venom produced in the poison glands, hemolytic and proteolytic substances have been found in the body and eggs of many spiders. These substances called variously epeiralysin, arachnotoxin, arachnolysin and epeiratoxin have been found only in gravid females, in their eggs and in newly-hatched spiderlings (Kaston, 1948).

MATERIALS AND METHODS

Our study dealt mainly on the identification and mass-rearing of spider species which caused araneidism. These spiders were subjected to conventional techniques used in spider taxonomy as follows:

Fixation of spiders:

The spiders were placed in jars containing Oudemans fixative solution (85 parts 70% ethyl alcohol, 5 parts glycerine and 5 parts glacial acetic acid). The preservative was changed after two days as it became diluted by the body fluids. The same preservative was used for permanent storage.

Observation and description:

The spiders were focused under the 150X Spencer binocular dissecting microscope. Both the qualitative and meristic features of the spiders were examined and described.

Identification of spiders:

Utilizing all the observed morphological features particularly the female genitalia or epigynum, the specific identities of the spiders were determined.

Photography and illustration:

The spiders were posed dry on a graphing paper and photographed. The fine details of the body structures were emphasized through line drawings with the aid of 20X stereoscopic Nikon microscope.

TAXONOMIC DESCRIPTIONS

Utilizing our devised identification schemes, the two species were found to belong to the same family, Family Araneidae. The spider from Los Baños was determined to be the giant orb-weaver, *Nephila pilipes* (Fabricius) (Figs. 1 and 2) whereas the spider from Cabanatuan City was the garden orb-weaving spider, *Neoscona minima* F.O.P. Cambridge (Figs. 3 and 4). *N. pilipes* was earlier reported to occur in rice fields (Barrion, 1980). *N. minima* is a new record to Philippine spider fauna.

The complete description of the two species are as follows:

Family .Araneidae

Genus: *Nephila*

Species:

Nephila pilipes (Fabricius) 1793.

Aranea longipes Fabricius 1781. Spec. Ins. 1. p. 545 (D ♀) (praeocc. Fuesslin 1775); Olivier 1789, Encycl. Method. 4 p. 234 (D ♀); Fabricius 1973, Ent. Syst. 2 p. 425 (D ♀).

A. maculata Fabricius 1793, Ent. Syst. 2 p. 425 (D ♀).

A. chrysogaster Walckender 1802. Faun. Paris 2 p. (♀).

A. sebae Walckenaer 1802. Faun. Paris 2 p. 55 (D ♀).

Epeira chrysogaster Walckenaer 1805. Tabl. Aran. p. 53 (N); 1841, Hist. Nat. Inst. Apt. 2 p. 92 (D ♀); Doleschall 1859, Act. Soc. Neer. Ind. 5 p. 27 T. 14 F.2; T.11 F.2 (D ♀).

N. maculata Leach 1815, Zool. Misc. 2 p. 134 T. 110 (D ♀); Thorell 1877, Ann. Mus. Civ. Genova 10 p. 455 (D ♀); McCook 1893 Amer. Spid. 3 p. 254 T. 23 F. 4 (D ♀); Simon 1894, Hist. Nat. Araign. 1(3) p. 755 F. 832, 833 (n ♀ ♂); Bosenberg and Strand 1906. Abh. Sen. Clembg. Grs. 30 (1-2) p. 193T. II F. 208 (D ♀); Dahl 1912, Mitt. Zool. Mus. Berlin (6) 1 p. 35, 52 (D ♀ ♂); Barrion 1980 M.S. Thesis p. 187 (D ♂).

N.fuscipes Walckenaer 1939, Die Arachniden 6 p. 136 TF. 528 (D ♀); 1871 Arachn. Austral. 1(1) p. 156 T. 13 F. 1 (D ♀); Thorell 1879, Ann. Mus. civ. Genova 13 p. 121 (D ♀).

E. fuscipes Walckenaer 1841, Fist Nat. Ins. Apt. 2 p. 97 (D ♀).

E. doreyana Walckenaer 1841, Hist. Nat. Ins. Apt. 2 p. 100 (D ♀).

E. caliginosa Walckenaer 1841, Hist. Nat. Ins. Apt. 2 p. 100 (D ♀).

N. ornata Adams 1841, Ann. Mag. Nat. Hist. 20 p. 291 (D ♀).

N. penicillum Doleschall 1857, Nat. Tijdschr. Ned. Ind. 13 p. 413 (D ♀).

E. kuhlii Doleschall 1859, Act. Soc. Neer. Ind. 5 p. 27 T.9 F.7 (D ♀).

E. harpyia Doleschall 1859, Act. Soc. Neer. Ind. 5 p. 28. T. 14 F. 1 (D ♀).

N. chrysogaster Cambridge 1871, Proc. Zool. Soc. London p. 620 T. 49 F. 3, 4 (D ♀ ♂).
Meta ornata L. Koch 1871, Arachn. Austral. 1(1) p. 134 T. 11 F. 6 (D ♂).
N. pecuniosa L. Koch 1871, Arachn. Austral. 1(1) p. 157 T. 13 F. 2 (D ♀).
N. aurosa L. Koch 1871, Arachn. Austral. 1(1) p. 160 T. 13 F. 2 (D ♀).
N. procera L. Koch 1871, Arachn. Austral. 1(1) p. 162 T. 14 F. 1 (D ♀).
N. sulphurosa L. Koch 1871, Arachn. Austral. 1(1) p. 163 T. 14 F. 2 (D ♀).
N. tenuipes L. Koch 1871, Arachn. Austral. 1(1) p. 165 T. 13 F. 5 (D ♀).
N. kuhlii Thorell 1887, Ann. Mus. Civ. Genova 125 p. 150 (D ♀); Pocock 1900, Faun. Brit. Ind.; Arachn. p. 218 (D ♀).
N. submaculata Strand 1906, Jahrb. Nassau. Ver. Nat. 59 p. 30 (D ♀).

♀: *Carapace* 5.0 mm long, 5.0 mm wide, 3.0 mm high; *Abdomen* 14 mm long, 5.0 mm wide, 4.0 mm high; *Total length* 19.0 mm.

Cephalothorax with rough outline, dark brown with fine black and white hairs along the arched margins, cervical groove distinct, narrow *pars cephalica* demarcated from wider *pars thoracica*, thoracic groove M-shaped; 8 heterogeneous eyes of similar sizes, anterior median eyes dark, all others hyaline, anterior lateral eyes a bit larger than the rest, anterior row recurved, posterior row slightly recurved to almost straight, lateral eyes set on small tubercules, area between lateral and median eyes depressed, median ocular quad wider than long, slightly narrower in front than behind, height of clypeus half the distance separating anterior median and anterior lateral eyes; chelicerae geniculate paturon black and robust, length equals head width, fangs black with red tips, 4 promarginal and 4 retromarginal teeth, lateral boss conspicuous, brown thick scopulae present; pedipalp simple, black with bristles and spines present all over and concentrated at apex; endites brown except yellowish serrula broad apically, brown scopulae very dense, labium black with reddish brown rebordered tip, longer than wide with bristles, mobile or free, basal end of labium shorter than frontal end of sternum; sternum black, longer than wide and tapers posteriorly; abdomen soft, longer than wide and tapers posteriorly, black with yellow spots on dorsum, lung covers reddish brown with transverse furrows, simple epigynum with small transverse lid-like plate covering the opening of the oviduct, a chitinized portion of cuticula of segment in front of epigastric furrow, convex scape at middle portion of atriolum covering opening of oviduct, spinnerets situated on a hump at lower portion of abdomen, separated from one another, anterior pair larger than posterior, colulus present; legs prograde, long and slender, black with narrow reddish brown bands, coxae yellow ventrally, fine dark hairs present all over; hair tufts on femur and tibia of legs I, II and IV, tarsi with 33 serrated claws. Leg formula I – II – IV – III.

Type-species: *Aranea longipipes* (Fabricius)

Leg Segment Measurements (mm) : (♀)

Leg no.	*Femur*	*Patella*	*Tibia*	*Metatarsus*	*Tarsus*	*Total*
I	10.0	2.0	8.0	13.00	2.0	35.00
II	7.0	2.0	6.0	9.00	2.0	26.00
III	5.0	1.0	4.0	1.75	1.0	12.75
IV	6.0	1.0	5.0	6.00	2.0	20.00

Specimen examined:

One adult female from Los Baños, Laguna submitted on February, 1985 by two residents of Los Baños, Laguna.

Distribution:

N. pilipes was found in Ceylon, India, China and Australia (Roewer, 1942-44). Its occurrence in the Philippines was first recorded by Mr. A.T. Barrion (1980).

Family: Araneidae

Genus: *Neoscona*

Species:

Neoscona minima F.O.P. – Cambridge F.O.P. Cambridge, 1904, Biologia Centrali. Americana Araneidae, 2:471, pl 44 Fig. 11, 12 ♀ ♂; Gertsch and Mulaik, 1936, American Mus. Novitates, 863: 20 Fig. 30 ♀; Kaston 1948, Bull. Connecticut Geol. Natur. Hist. Surv. 70:245, Fig. 751, 776 ♀ ♂; Bonnet 1958, Bibliographia Araneorum, 2:3058, New Synonymy; F.O.P. Cambridge 1904, Biol. Centrali Americana, Arachn. Aran., II .471, pl. xliv, f. 11-12. ♀ ♂

♀: *Carapace:* 4.50 mm long, 3.50 mm wide, 3.25 mm high, *Abdomen:* 7.0 mm long, 8.0 mm wide, 6.0 mm high. *Total length:* 11.5 mm.

Cephalothorax light brownish orange, cervical groove present, narrow *pars thoracica*, thoracic groove deep and vertical, radial furrows distinct, fine white bristles present all over; 8 heterogeneous ocelli, all dark, medians larger than laterals, laterals nearer to each other than to their respective medians, anterior row slightly procurved, posterior row slightly recurved; chelicerae geniculate, paturon brownish red, fangs with dark brown base and red tip, 4 promarginal teeth, 3 retromarginal teeth, lateral boss present; pedipalp simple, lightbrownish red in color, light and dark bristles present all over, more bristles concentrated at tip, few spines also present at tip; endites squarish, light brownish red, brown scopulae present, labium light brownish red with white tip, wider than long and rebordered, basal end of labium shorter than frontal end of sternum; sternum light brown with cream median longitudinal portion, longer than wide and pointed at caudal end; abdomen soft, subtriangular, globular, light brown colored with folium at angles outlined in brown, fine white bristles present all over, lung covers marked with transverse furrows; epigynum well developed, simple, tongue-like with chitinous free scape with sides approximately parallel protruding from atriolum pointed toward spinnerets, small dots set below lung covers and above spinnerets; spinnerets brown fitting close to one another; colulus black, elongate and pointed; legs prograde, light brownish red, prolateral spines on femur, patella, tibia and metatarsus, fine light bristles present all over leg area, tarsi with two black claws at tip. Leg formula: I – II – IV – III.

Type-species: *Neoscona arabesca* (Walckenaer)

Leg Segment Measurements (mm) : (♀)

Leg no.	*Femur*	*Patella*	*Tibia*	*Metatarus*	*Tarsus*	*Total*
I	5.0	2.00	4.0	3.0	3.00	17.00
II	5.0	2.00	4.0	4.5	1.00	16.50
III	4.0	1.25	2.5	2.5	1.00	11.75
IV	5.0	2.00	4.0	4.0	1.25	16.25

Specimen examined:

One adult female from Cabanatuan City submitted on June 5, 1986 by Dr. Patricia Grace E. Dañgo, resident physician of the Hospital of the Infant Jesus, Sampaloc, Manila.

Remarks:

Descriptions and illustrations were based on one female specimen identified by Dr. A.A. Barrion. Its occurrence in the Philippines is a new record.

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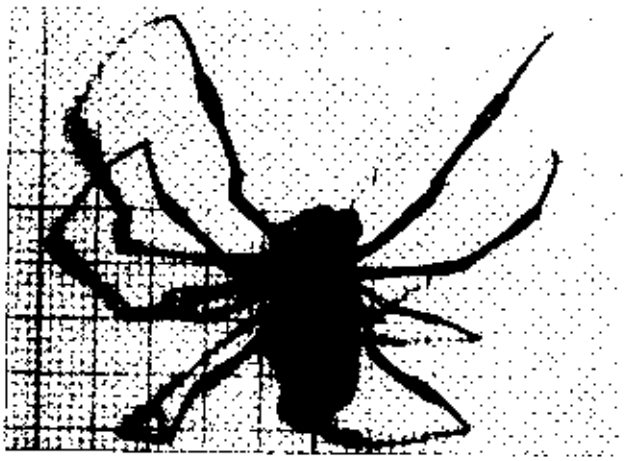

Fig. 1. Female Nephila pilipes *(Fabricius).*

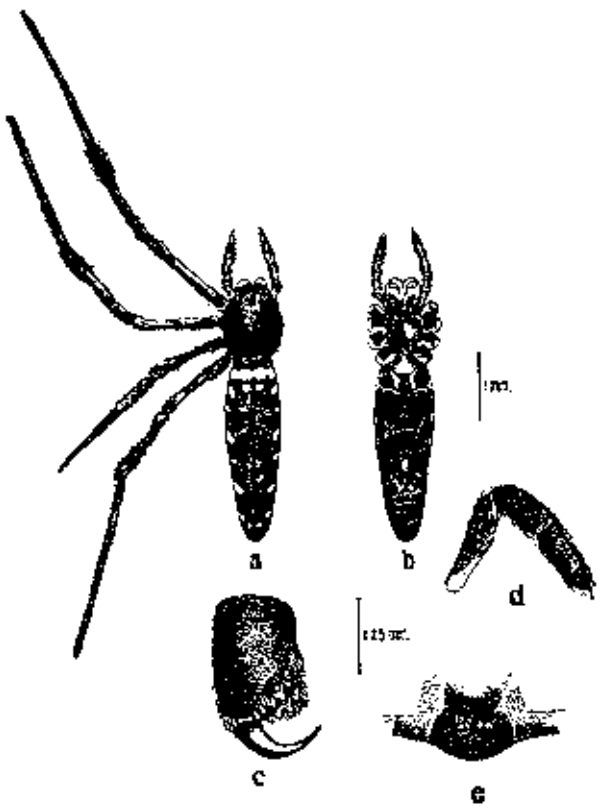

Fig. 2. Nephila pilipes *(Fabricius), female dorsal view (a), ventral view (b), chelicera (c), pedipalp (d), and epigynum (e).*

Fig. 3. Female Neoscona minima *F.O.P. Cambridge.*

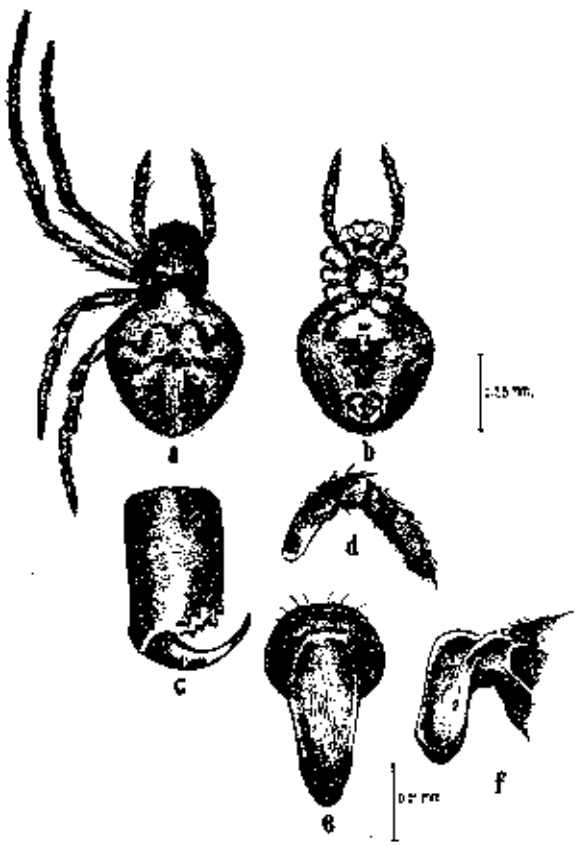

Fig. 4. Neoscona minima *F.O.P. Cambridge, female dorsal view (a), ventral view (b), chelicera (c), pedipalp (d), and epigynum (e and f).*

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Length

meter	m
millimeter	mm
centimeter	cm

Volume

liter	L
milliliter	mL
cubic meter	m^3
Energy and Work	KJ

kilojoule (replace calorie in dietetics)

Mass

kilogram	kg
gram	g
ton (metric ton)	t
milligram	mg

Time (same units used in both Metric and English System)

day	d
hour	h
minute	min
second	s

Amount of substance

mode	mole

Temperature

degree celsius	°C

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